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COOPERATIVE NUCLEASE ACTIVITY OF THE MRE11/RAD50/XRS2 COMPLEX AND SAE2 DURING DNA DOUBLE-STRAND BREAK REPAIR

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DNA double-strand breaks (DSBs) are lethal in eukaryotic cells if left unrepaired. In *Saccharomyces cerevisiae* the Mre11/Rad50/Xrs2 (MRX) complex is required for repair of DSBs through homologous recombination and non-homologous end joining. Although Mre11 complexes exhibit 3'→5' exonuclease activity and endonuclease activity on DNA hairpin and single-stranded DNA overhang substrates in vitro, the role of the MRX complex in homologous recombination in vivo is not well understood. It has been shown to be specifically required for the processing of protein-conjugated DNA ends at DSBs during meiosis and hairpin-capped DSBs in mitotic cells and has been suggested that the Mre11 nuclease functions to remove damaged DNA ends. Recently, the Sae2 protein has been demonstrated to be involved in hairpin-capped DSBs and

DNA end processing along with MRX in vivo. However, the Sae2 protein has no known homologs outside of fungi and no obvious motifs to suggest the function(s) of the Sae2 protein. We have purified recombinant Sae2 and MRX and report that the Sae2 protein itself is a single-stranded DNA endonuclease. The Sae2 protein stimulates the 3'→5' exonuclease activity of the MRX complex. Also, the MRX complex can stimulate Sae2 nuclease activity to cleave ssDNA adjacent to DNA hairpin structures. The Sae2 protein also binds independently to double-stranded DNA and forms higher order protein-DNA complexes with MRX. These results provide biochemical evidence for functional cooperatively between MRX and Sae2 on DSBs and hairpin-capped DNA ends.

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CHAPTER 1: INTRODUCTION

DNA DOUBLE-STRAND BREAK REPAIR

Repair of DNA double strand breaks (DSBs) is essential for cell survival, and failure to repair such damage can lead to cell death. Two pathways, homologous recombination (HR) and nonhomologous end joining (NHEJ), are used to repair DNA DSBs (Krogh and Symington, 2004). The HR form of recombinational repair utilizes homologous regions in the intact chromosome or sister chromatid as the template to repair the broken DNA regions. As a result, this process is error-free and is the preferred pathway in *Saccharomyces cerevisiae* to repair broken DNA molecules. Nonhomologous end joining on the other hand is error-prone and is less preferred. NHEJ occurs in yeast when homologous DNA sequences are absent and this can potentially lead to deletions in genomic sequences.

Homologous recombination utilizes homologous DNA sequences to yield error-free repair products. This process initially involves the resection of 5' strands at sites of DNA damage, yielding long 3' single-strand overhangs (Krogh and Symington, 2004) (Figure 1.1A). Despite extensive efforts, the protein(s) responsible for the initial break-processing steps are still not known. The single-stranded DNA regions can span from hundreds to a few thousand nucleotides in *S. cerevisiae* (Krogh and Symington, 2004) and are used to invade homologous

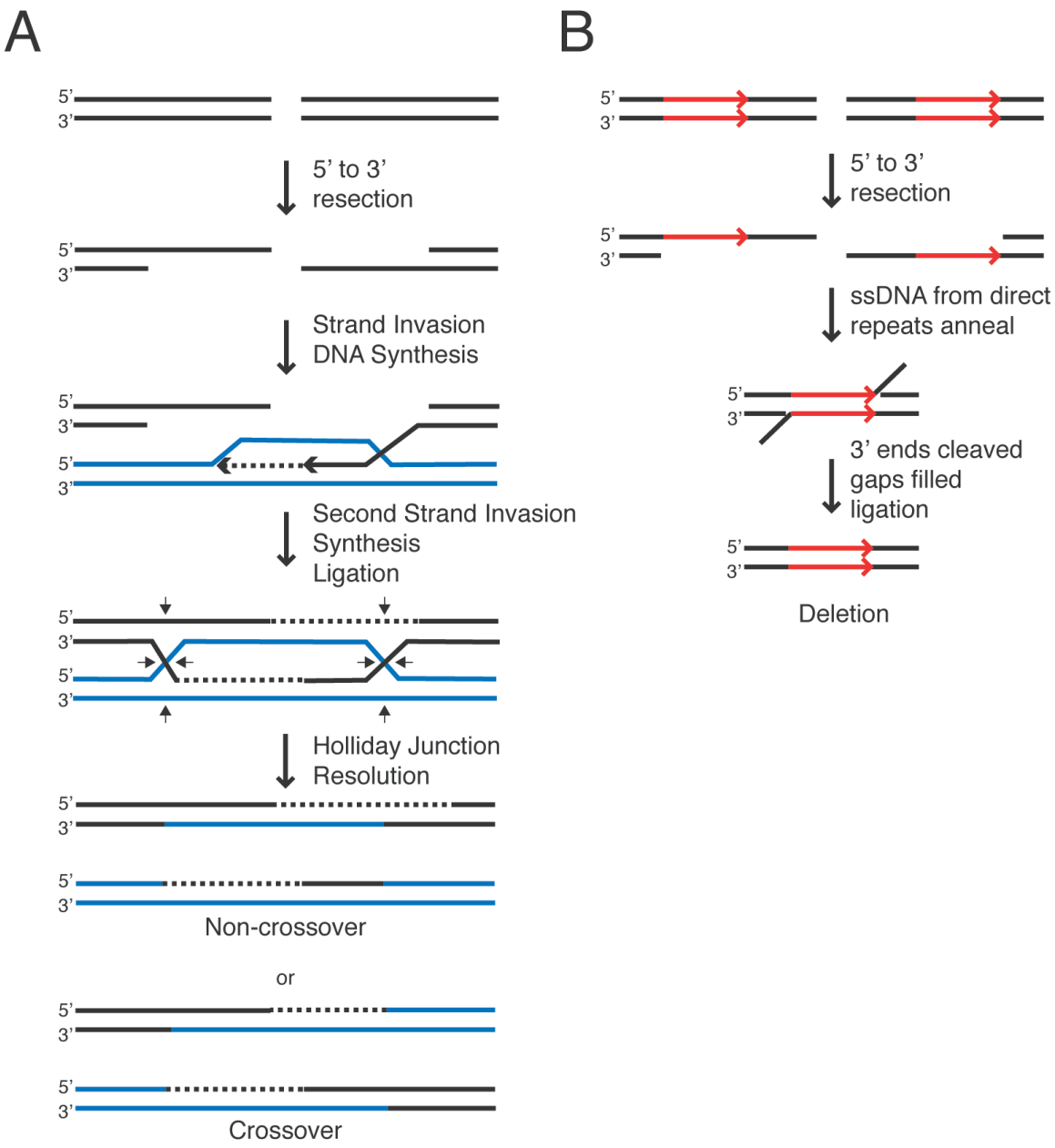

Figure 1.1 Models for repair of DNA double-strand breaks. A) Homologous recombination is initiated by invasion of 3' ssDNA tail formed by resection of a DSB, into a homologous DNA template. Holliday junctions are formed after DNA synthesis and second strand invasion. Resolution of Holliday junctions result can result in crossover or non-crossover products. B) Single-strand annealing occurs between two direct repeats near a DSB. After resection the homologous sequences anneal. Excess 3' ssDNA tails are removed, gaps are filled and ligated. (Figure adapted from Krogh and Symington 2004)

DNA regions that can be used as a template for repair. DNA synthesis extends the invading strands to form strand invasion intermediates known as double-Holliday-junctions, which are resolved to form crossover or non-crossover products (Krogh and Symington, 2004).

RAD52 EPISTASIS GROUP OF GENES

Members of the *RAD52* epistasis group are essential for homologous recombination in *S. cerevisiae*, and deletion of *RAD52* blocks all forms of homologous recombination events. Gene products of *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RAD50*, *MRE11* and *XRS2* belong to the *RAD52* epistasis group and deletion of these genes confers sensitivity to ionizing-radiation (IR) as well as many radiomimetic compounds (Symington, 2002). The Rad51 protein, similar to RecA in prokaryotes, forms nucleoprotein filaments on 3' single-stranded DNA tails to promote strand exchange between homologous DNA molecules (Ogawa et al., 1993). Interaction between Rad52 and Rad51 stimulates the formation of the Rad51 nucleoprotein filament while several other factors including Rad54, Rad55 and Rad57 promote strand invasion. Most of the gene products in the *RAD52* epistasis group are only involved in homologous recombination, except Mre11, Rad50 and Xrs2 which form a subgroup that is involved in both homologous recombination and NHEJ (Symington, 2002). Mre11, Rad50 and Xrs2 form a heterotrimeric complex and studies have

revealed that it plays a critical role in meiotic DSB formation and processing, NHEJ, telomere maintenance, DNA damage checkpoints, and homologous recombination in mitotic cells (Krogh and Symington, 2004; Symington, 2002).

STRUCTURAL FEATURES OF THE MRE11/RAD50/XRS2 COMPLEX

The Mre11 and Rad50 components of the MRX complex are highly conserved throughout all organisms and are involved in both homologous recombination and NHEJ. Xrs2 in yeast and Nbs1 in mammals are functional homologs and constitute the third component of the complex that is only present in eukaryotes. The MRX complex is implicated in DNA end processing at DSBs and is also involved in checkpoint signaling after DNA damage (Krogh and Symington, 2004).

In *S. cerevisiae*, the 78kDa Mre11 protein contains four phosphoesterase motifs in the N-terminal domain (Figure 1.2) with sequence similarity to the evolutionarily conserved family of nucleases that include the *E. coli* SbcD and human Mre11 proteins. Point mutations in any of the four phosphoesterase motifs render the Mre11 protein nuclease-deficient (Figure 1.2) (Krogh et al., 2005; Nairz and Klein, 1997). The SbcD, yeast Mre11, and human Mre11 proteins all exhibit manganese-dependent 3'→5' exonuclease activity, endonucleolytic activity at the tip of DNA hairpin structures and single-

strand/double-strand DNA junctions (Connelly et al., 1998; Paull and Gellert, 1998; Trujillo and Sung, 2001).

Rad50 is a 152kDa protein in yeast, and like Mre11 is conserved through all organisms. It belongs to the ABC transporter superfamily of ATPases that also include Structural Maintenance of Chromosomes (SMC) proteins. Rad50 contains Walker A and Walker B motifs at the N-terminus and C-terminus, respectively, which function as an ATPase when the domains are brought together (Figure 1.2). The Walker motifs are separated by long heptad repeats that form a coiled-coil domain, and a zinc-hook motif, which is at the center of the molecule. The coiled-coil domain folds intramolecularly such that the N-terminus and C-terminus form the catalytic site while the zinc-hook mediates dimerization with another Rad50 molecule. The crystal structure of the catalytic domains of Pyrococcus furiosus Rad50 reveals that ATP-binding facilitates dimerization of two Rad50 molecules in a head-to-tail orientation (Hopfner et al., 2000).

Xrs2 in *S. cerevisiae* and Nbs1 in mammals are only found in eukaryotes and are functional homologs. Xrs2 and Nbs1 contain a fork-head-association (FHA) domain at the N-terminus (Figure 1.2). The FHA domain is thought to regulate interactions between phosphorylated proteins. The Xrs2 and Nbs1 protein also contains two breast cancer carboxy-terminal (BRCT) domains next to the FHA domain (Becker et al., 2006; Featherstone and Jackson, 1998).

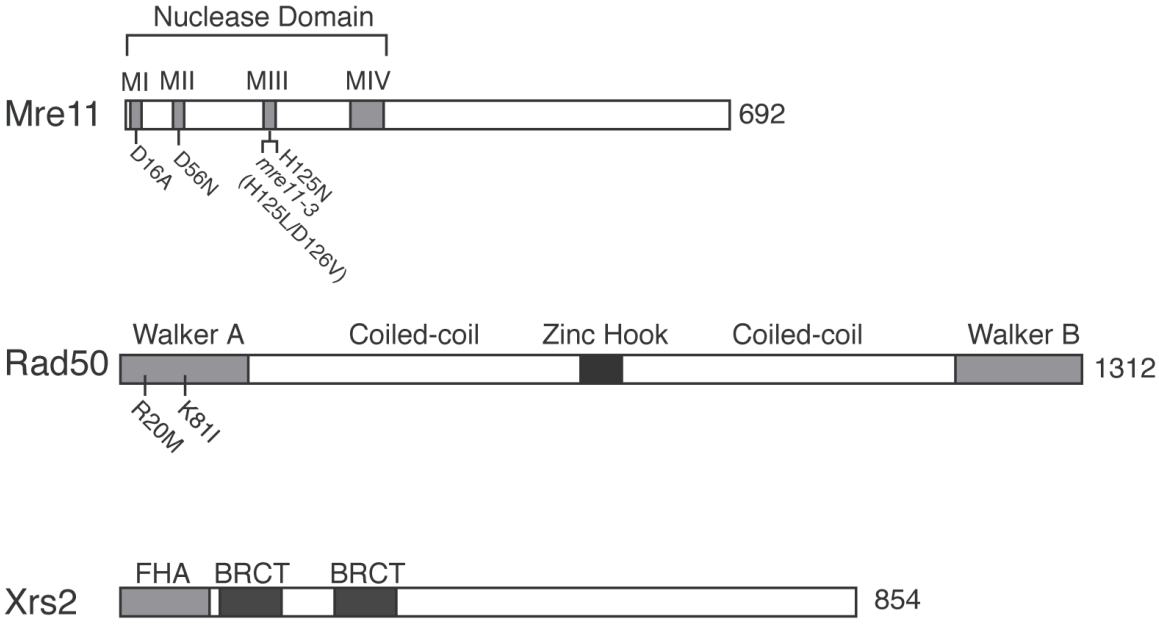

Figure 1.2 Schematic representations of Mre11, Rad50 and Xrs2 proteins. The Mre11 nuclease domain contains four phosphodiesterase motifs (I-IV). Mre11-D16A, Mre11-D56N, Mre11-3, and Mre11-H125N protein are nuclease-deficient in vitro. Rad50 contains a Walker A and Walk B motif separated by two coiled-coil and one zinc-hook domain. Rad50-R20M and Rad50-K81I are *rad50S* mutants. Xrs2 contains an FHA and two BRCT domains at the N-terminus. (Figure adapted from Symington 2002)

Both the FHA and BRCT domains are phospho-specific protein-protein interaction motifs that are found in several DNA damage checkpoint proteins (Symington, 2002). The Xrs2 and Nbs1 proteins are primarily implicated in cell cycle checkpoint signaling mediated by MRN(X) and ATM(Tel1) (D'Amours and Jackson, 2001) but the role of Xrs2 in the context of DSB processing and repair is not yet clearly understood.

THE MRE11 COMPLEX IN VIVO

In *Escherichia coli* the SbcD and SbcC proteins, homologs of Mre11 and Rad50, respectively, were shown to be involved in DNA hairpin resolution in vivo (Connelly et al., 1998). Long palindromic repeats have the potential to form cruciforms or hairpin structures during DNA replication and are removed from the genome in wildtype cells; however, in SbcD and SbcC deletion strains, palindromic repeats are stabilized (Cromie et al., 2000). This suggests that the SbcD/SbcC complex is involved in recognizing and resolving DNA hairpin structures.

Null strains of any of the components of the MRX complex in *S. cerevisiae* confer extreme sensitivity to ionizing radiation (IR) and other DNA damaging agents, defects in telomere maintenance, NHEJ, and meiotic DSB repair (Krogh and Symington, 2004). Genetic studies have revealed that null strains of *mre11, rad50* and *xrs2* cause gross chromosomal rearrangements (Chen and Kolodner,

1999) and exhibit hyper-recombination phenotypes (Symington, 2002). Deletion of any of the MRX components delays resection of the DSB ends; however, the deletion strains still complete homologous recombination with nearly wildtype efficiency (Llorente and Symington, 2004; Moreau et al., 1999). NHEJ is also defective in *Δmre11, Δrad50,* and *Δxrs2* strains (Moore and Haber, 1996; Zhang and Paull, 2005). End joining between linear DNA ends is reduced as severely as in strains lacking Ku70 and Ku80 proteins, essential NHEJ factors (Moore and Haber, 1996). *mre11* nuclease-deficient mutants do not show a defect in NHEJ, suggesting the MRX complex is required but not the nuclease activity of Mre11.

In contrast to *E. coli* and *S. cerevisiae* homologs, loss of any of the components of the Mre11 complex is lethal in vertebrate cells (Luo et al., 1999; Xiao and Weaver, 1997; Zhu et al., 2001). In mice, the Mre11, Rad50, and Nbs1 genes are thus essential for early embryonic development.

ROLE FOR THE MRX COMPLEX IN DSB REPAIR

Genetic studies suggest that the MRX complex stabilizes sister chromatid interactions to support homologous recombination (Bressan et al., 1999). Wildtype haploid cells are 100-fold more sensitive to IR during G_1 phase compared to the G_2 phase of the cell cycle, suggesting that during G_2, homologous recombination is more efficient when sister chromatids are present. Survival of *Δmre11* strains after IR exposure was similar during both G_1 and G_2

phases, suggesting that the MRX complex is required for sister chromatid-based homologous recombination DSB repair. Wildtype diploid strains exhibit little sensitivity to IR during either G_1 and G_2 phases because during both cell cycle phases, homologous chromosomes are available to act as a donor for homologous recombination. Diploid *Δmre11* strains also exhibit increased survival after exposure to IR in comparison to haploid strains, suggesting that the MRX complex is not required for recombination between homologs.

SEPARATION OF FUNCTION MUTANTS OF THE MRX COMPLEX

Several *rad50* mutants were isolated by Kleckner and colleagues, who found that meiotic recombination was initiated but was blocked in intermediate stages during recombination (Alani et al., 1990). In contrast, *Δrad50* mutant strains were unable to initiate meiotic DSB formation and therefore recombination did not occur. On further investigation of the meiotic DSBs in *rad50S* strains, it was found that a protein, Spo11, was covalently linked to the 5' DNA strands on the DSB ends (Keeney et al., 1997). These *rad50S* mutants are only mildly sensitive to MMS when compared to wildtype and *Δrad50* strains. The nonnull *rad50* mutants confer more severe meiotic phenotypes compared to their mitotic phenotypes and therefore are termed "separation of function" or "*rad50S*".

From the *rad50S* phenotype in yeast and experiments using many other organisms, the Spo11 protein is now known to be the protein that initiates meiotic

recombination by catalyzing DSB formation during prophase of meiosis I (Krogh and Symington, 2004; Paques and Haber, 1999). The Spo11 protein, a homolog of Top6A from Sulfolobus shibatae and a type IIB topoisomerase, is thought to cleave DNA by forming a 5' phophotyrosyl linkage at DNA ends (Keeney et al., 1997; Keeney and Neale, 2006). The *rad50S* phenotype suggests that, unlike typical topoisomerases, Spo11 is not able to religate the DNA ends and must therefore be removed to proceed with homologous recombination. *mre11S* mutants were also identified and exhibited phenotypes similar to the *rad50S* mutants (Nairz and Klein, 1997). Taken together, this evidence suggests that MRX is involved in the removal of protein adducts from DSBs during meiosis.

ROLE OF MRE11 NUCLEASE ACTIVITY IN VIVO

The phenotype of the *rad50S* and *mre11S* mutants suggested that Mre11 nuclease activity may be responsible for generating 3' ssDNA tails prior to homologous recombination during meiosis. Consistent with this hypothesis, *mre11* nuclease-deficient mutants were found to exhibit a *rad50S* phenotype in meiosis (Moreau et al., 1999). However, the Mre11 protein exhibits exonuclease activity in the 3'→5' direction in vitro, which is the opposite polarity required for the generation of 3' ssDNA tails. In addition, *Δmre11* and nuclease-deficient *mre11* mutant strains are capable of repairing DSBs by homologous recombination in vegetative cells. Furthermore, *mre11-3, mre11-N113S* and

mre11-D56N nuclease-deficient mutants exhibited similar rates of 5'→3' resection in comparison to wildtype strains. However, *Δmrx* strains do exhibit a 1 to 2 hour delay in resection (Ivanov et al., 1994). At this point the process of DNA end resection is not understood and it is possible that several enzymes may contribute to this process in vegetative cells.

The MRX complex plays a critical role in the NHEJ pathway. When a DSB is introduced in the MAT locus and homologous recombination is blocked by deletion of a homologous donor sequence, repair is dependent on NHEJ (Moore and Haber, 1996). NHEJ-mediated repair of plasmids transformed into yeast is also completely dependent on the MRX complex (Boulton and Jackson, 1998). However, the *mre11* nuclease-deficient mutants, *mre11-3, mre11-4, mre11-58S* and *mre11-N113S*, all rescued *Δmre11* strains nearly to wildtype levels in this NHEJ assay (Lee et al., 2002). In addition, the *mre11* nuclease-deficient mutant *mre11-H125N*, fully complemented *Δmre11* strains for the joining of mismatched DNA ends as well as complementary ends (Zhang and Paull, 2005), suggesting that Mre11 nuclease activity is not required for NHEJ, even though the MRX complex is required.

Several *mre11* nuclease-deficient strains only have intermediate sensitivity to IR and MMS in comparison to *Δmre11* strains. The *mre11-4* and *mre11-58S* mutant strains are as sensitive to IR and MMS as *Δmre11* strains (Lee et al., 2002); however, two-hybrid and coimmunoprecipitation assays have

showed that these two mutant Mre11 proteins do not interact with Rad50 (Lee et al., 2002). This suggests that complex formation is critical for MRX function in meiotic DSB repair and less so for the nuclease activity of the protein. The intermediate sensitivity of the *mre11* nuclease-deficient strains to DNA-damaging agents does suggest a requirement for Mre11 nuclease activity on a subset of certain DSBs. Both IR and MMS have the potential to create DSBs with DNA-protein or DNA adducts so it is possible that the Mre11 nuclease activity is required for removal of DNA adducts as a result of IR or MMS. Moreover, in mitotic cells, redundant nucleases such as Exo1 and Rad27 may be able to process damaged DNA ends in the absence of the Mre11 nuclease activity (Moreau et al., 2001).

REDUNDANT NUCLEOLYTIC PATHWAYS IN MITOTIC CELLS

Although Mre11 nuclease activity is required for meiosis-specific DSB repair, the nuclease activity is only partially required for mitotic DSB repair which suggests there are other nucleases present which can compensate for the loss of Mre11 nuclease activity. Overexpression of Exo1, a 5'→3' exonuclease, was shown to rescue the resection rate and suppress sensitivity to IR in *Δmre11* mutant strains (Lee et al., 2002), suggesting that MRX may be involved in resection but other nucleases can compensate for the loss of the Mre11 nuclease activity. Although, since wildtype levels of resection are not observed,

these other nucleases cannot compensate for all of the MRX functions. Also, *Δexo1 mre11-H125N* double mutants exhibited normal mating type switching and were more resistant to IR than a *Δmre11* single mutant (Moreau et al., 2001). This suggests loss of Exo1 and Mre11 nuclease activities can be compensated by other nucleases but not the loss of the MRX complex.

One of the other nucleases implicated in these DSB repair functions is Rad27(Fen1), a 5' flap endonuclease involved in Okazaki fragment processing during replication. Deletion of *RAD27* causes increased sensitivity to DNA damaging agents, increased spontaneous recombination, and instability of repetitive DNA (Moreau et al., 2001). Deletion of any of the *RAD52* epistasis group genes in a *Δrad27* background show synthetic lethality. The *rad50S, mre11S* and *Δsae2* mutants also are synthetic lethal in a *Δrad27* background (Moreau et al., 2001). This suggests homologous recombination is the major pathway used to bypass replication defects in the *Δrad27* mutant strains.

MRX IN VITRO

Several studies have been performed to characterize the Mre11 complex in vitro. Recombinant *E. coli, S. cerevisiae* and human Mre11 complexes were purified and tested for nuclease activity on various biologically relevant DNA substrates. The recombinant SbcC/D complex was shown to cleave DNA hairpin structures at the hairpin tip in an ATP-independent and manganese-dependent

manner (Connelly et al., 1998), in agreement with in vivo results showing the SbcC/D complex is required to resolve palindromes in vivo (Leach, 1994). Later, the SbcC/D complex was also found to exhibit 3'→5' exonuclease activity, in an ATP-dependent manner (Connelly et al., 1999).

The human Mre11/Rad50/Nbs1 complex (MRN) was also purified and tested in vitro for similar activities (Paull and Gellert, 1998). Similarly to the SbcD protein in *E. coli*, human Mre11 possesses manganese-dependent 3'→5' exonuclease activity on 3' recessed and blunt-ended substrates. Mre11 in complex with Rad50 increases the exonuclease activity in addition to cutting DNA hairpin structures asymmetrically at the loop (Paull and Gellert, 1998). The human MRN complex also exhibits endonuclease activity on 3' ssDNA at single-strand/double-strand DNA junctions (Paull and Gellert, 1998).

Nuclease activity of the yeast MRX complex is also dependent on manganese, similar to the *E. coli* SbcCD and the human MRN complexes (Trujillo et al., 2003; Trujillo and Sung, 2001). The yeast Mre11 protein exhibits 3'→5' exonuclease activity, which is stimulated in the presence of the Rad50 protein and ATP (Trujillo and Sung, 2001). In vitro nuclease assays demonstrated that yeast MR complexes behaved similarly to human and *E. coli* complexes. In fact, the recombinant yMR complex also exhibited endonucleolytic activity on DNA hairpin tips (Trujillo and Sung, 2001). Hairpin cleavage was further increased in the presence of the Xrs2 protein. The Xrs2 protein has been

shown to target the MR complex to DNA ends, therefore Xrs2 may play a critical role in targeting the MRX complex to sites of DNA damage in the cell (Trujillo et al., 2003).

IDENTIFICATION OF SAE2

The *SAE2/COM1* gene was independently identified by Prinz et al. and McKee et al. and shown to be required for meiotic DSB repair (McKee and Kleckner, 1997; Prinz et al., 1997). Separate mutational screens were performed to screen for mutants that had viable spores in the absence of Spo11 but were inviable in the presence of Spo11 in order to identify genes involved in early stages of meiotic recombination. Furthermore, the *Δsae2* sporulation phenotype was identical to the previously discovered separation of function mutant, *rad50S* (Alani et al., 1990). *Δsae2* and *rad50S* mutant strains were blocked in meiotic recombination and Spo11-conjugated DNA breaks accumulated in these strains. The MRX complex, Sae2 and several other proteins are now known to be involved in meiotic DSB initiation, recombination, and repair of DSBs.

SAE2 ACTIVITY IN VEGETATIVE CELLS

The Sae2 protein was also found to function in vegetative cells. Rattray et al. designed an in vivo screen to identify mutants that decreased recombinational fidelity during DSB repair. A site-specific DSB was introduced between inverted repeats, leading to intrachromosomal homologous recombination between the

repeats (Figure 1.3). Errors in recombination resulted in duplication of the region between the inverted repeats. Recombinational errors between the inverted repeats after DSB introduction increased in *Δsae2* strains about 10-fold (Rattray et al., 2001). The nuclease-deficient mutant *mre11-H125N* and *rad50S* mutant *rad50-K81I* both exhibited similar defects in homologous recombination at the DNA break (Rattray et al., 2001). This suggests the MRX and Sae2 complexes are functioning together to repair the DSB. Analysis of the recombinational products in *Δsae2* strains revealed small inverted repeats present in the duplicated DNA sequence. It has been suggested that after the introduction of the DSB, the 3' ssDNA end folds back on itself at the small inverted repeats, creating a hairpin structure at the end. If the hairpin structure is not resolved, DNA synthesis will initiate from the 3' end and duplicate the DNA containing the hairpin. Multiple gene amplifications can occur if the small inverted repeats are not resolved.

Lobechev et al. also described the role of the MRX and Sae2 complexes in repair of DSBs resulting in hairpin-capped ends (Lobachev et al., 2002). Large inverted repeats were inserted into the *LYS2* gene on one chromosome while another *LYS2* gene on another chromosome was deleted at the 5' region (Figure 1.4). Just as in *E. coli*, these inverted repeats in yeast can extrude to form a cruciform and lead to gene amplification and rearrangement. The cruciform is cleaved, in a MRX-independent manner, at the base leading to hairpin-capped

DNA ends (Lobachev et al., 2002). The MRX and Sae2 complexes were required to remove the hairpin-capped ends to allow for homologous recombination between the two partial *LYS2* genes. Loss of any of the MRX components or of Sae2 complexes led to gene amplification.

TEL1/MEC1 PHOSPHORYLATION OF THE SAE2 PROTEIN

The Sae2 protein was found to be phosphorylated in response to DNA damage. Phosphorylation of Sae2 was absent in a *Δmec1Δtel1* double mutant as well as in a *Δrad50* single mutant, suggesting that Sae2 phosphorylation is entirely dependent on Mec1/Tel1, homologous to DNA damage signaling kinases ATR/ATM in mammalian cells. In this study, 5 putative (S/T)Q phosphorylation sites, S73, T90, S249, T279, and S289, in Sae2 were mutated to alanine, Sae2(5A), and studied in vivo. Mutation of all five putative phosphorylation sites to alanine blocked all Sae2 phosphorylation and increased sensitivity of the mutant strains to MMS (Baroni et al., 2004).

Phosphorylation of Sae2 has also been linked to the involvement of the Sae2 protein in negative regulation of cell cycle arrest after DNA damage. Rad53 is a protein kinase that is phosphorylated by Mec1/Tel1 to activate cell cycle arrest after DNA damage (Gardner et al., 1999). Used as an indicator of cell cycle arrest, Rad53 is phosphorylated in wildtype cells during checkpoint

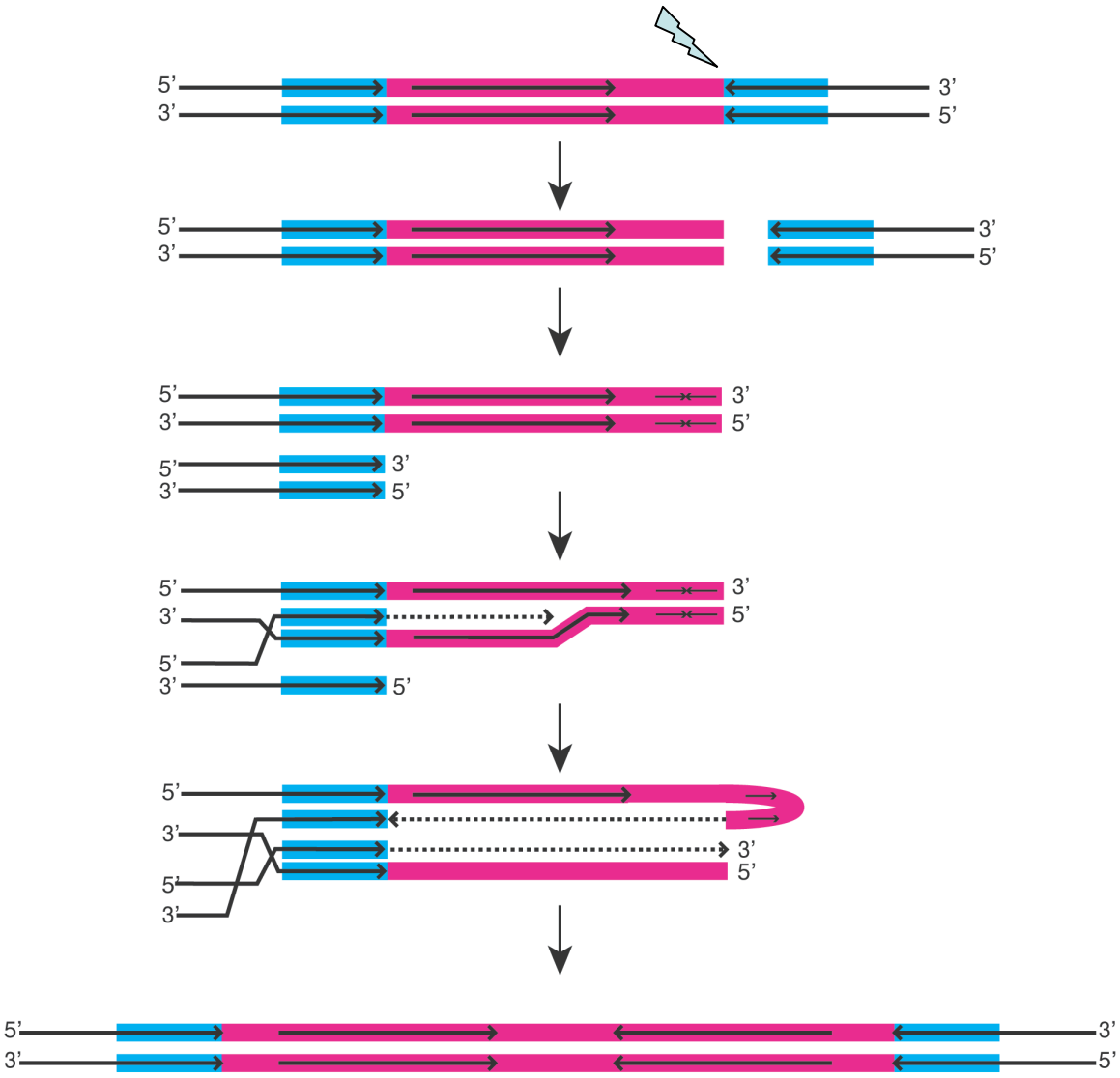

Figure 1.3 Model for gene amplification between inverted repeats. Formation of a DSB between inverted repeats can lead to strand invasion between the repeats. Foldback of a free 3’ end because of small (4-9 nucleotide) inverted repeats can prime DNA synthesis. Gaps are filled, leading to gene amplification. (Adapted from Rattray et al. 2006)

arrest and then dephosphorylated during recovery from the checkpoint. In both *Δsae2* and *sae2(5A)* mutants, Rad53 is phosphorylated indicating that cell cycle arrest has occurred, however, phosphorylated Rad53 is stabilized and the cells remain arrested (Baroni et al., 2004). Conversely, overexpression of wildtype Sae2 decreases Rad53 phosphorylation after DNA damage as well as cell cycle arrest (Clerici et al., 2005a). This data suggests that the Sae2 protein is involved in cell cycle arrest and checkpoint recovery after DNA damage.

DNA DOUBLE STRAND BREAK FOCI

Lisby et al. investigated the requirements and spatiotemporal order of localization of various DSB repair proteins (Lisby et al., 2004). The Tel1 protein and the MRX complex are the first among the *RAD52* epistasis group detected at the DNA break. This is similar to mammalian cells in which the ATM kinase (Tel1 homolog) colocalizes with MRN to sites of DNA breaks (Paull et al., 2000). The Sae2 protein was also found to localize to DSBs shortly after the MRX and Tel1 complexes. Sae2 foci are still present in *Δtel1Δmec1* double mutant strains and also in *Δmre11* strains, showing that Sae2 is not dependent on phosphorylation or MRX for DNA association (Lisby et al., 2004). Mre11 foci were still present without Sae2, although deletion of *SAE2* delayed Mre11 release from the DNA break. Removal of Mre11 from DSB foci was also delayed

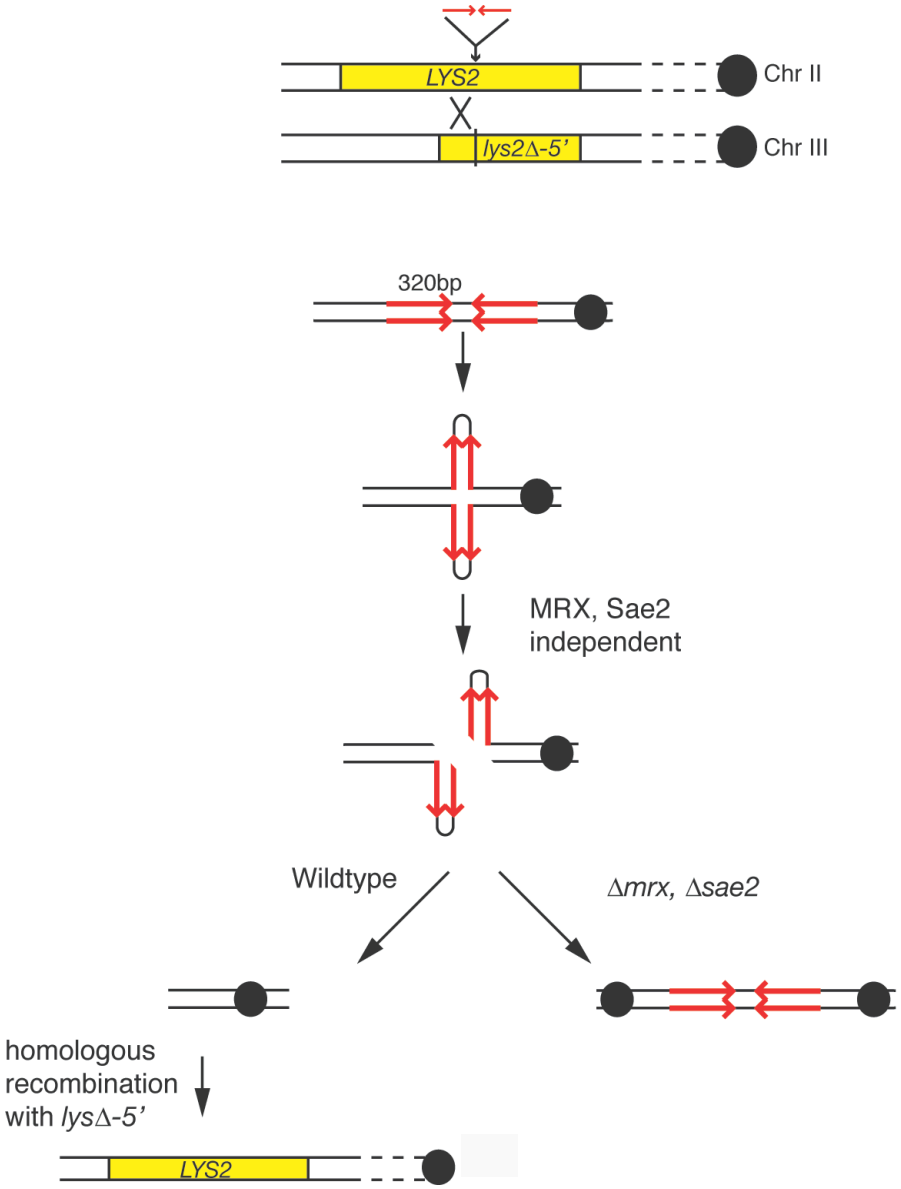

Figure 1.4 Model for gene amplification at cruciform structures. A) 320bp inverted repeats were embedded into the *LYS2* gene. B) Inverted repeats extrude forming a cruciform structure. The base of the cruciform structure is cleaved in an MRX and Sae2-independent manner. In *Δmrx* and *Δsae2* strains the hairpin-capped end leads to DNA synthesis and duplication of the palindrome. In wildtype cells MRX and Sae2 remove the hairpin structure, resulting in homologous recombination. (Adapted from Lobachev et al 2002)

in *rad50S* and *sae2(5A)* strains, suggesting that DNA end processing activity of the MRX and Sae2 complexes are required for efficient release of the complexes from the DNA break (Baroni et al., 2004; Lisby et al., 2004). Interestingly, overexpression of wildtype Sae2 actually leads to Mre11 foci releasing more rapidly than in wildtype cells (Clerici et al., 2005a). Release of Sae2 foci was also delayed in *Δmre11, mre11-H125N* and *mre11-D56N* nuclease-deficient strains (Lisby et al., 2004). This data suggest MRX localizes to the DNA break site first and Sae2 localizes shortly afterwards, although if processing at the break site is hindered by loss of nuclease activity or Sae2 itself then release of MRX from the DSB site is delayed.

THE SAE2 PROTEIN IS INVOLVED IN SINGLE-STRAND ANNEALING

Single strand annealing (SSA) is a homologous recombination repair pathway that is dependent on Rad52 (Vaze et al., 2002). During SSA, 3' ssDNA tails with homologous DNA sequences flanking a DSB site anneal and the resulting ssDNA flaps are removed before DNA synthesis and ligation of the DSB (Figure 1.1B). It was recently demonstrated that loss of the Sae2 protein diminishes DSB repair by SSA, as does a *rad50S* mutantion (Clerici et al., 2005b). Interestingly, overexpression of Sae2 in a *rad50S-(K81I)* background partially suppresses the SSA defect seen in this strain (Clerici et al., 2005b). This suggests that Sae2 plays a critical role in SSA and that a possible

explanation for defects in *rad50* and *mre11* separation of function mutants may be a diminished interaction between the MRX and Sae2 complexes.

For SSA to occur efficiently, both DNA ends at a DSB must be held in close proximity. Deletion of MRX components or of Sae2 was shown to decrease end bridging between two DSB ends in vivo (Clerici et al., 2005b; Kaye et al., 2004; Lobachev et al., 2004). Interestingly, the *rad50S* mutant exhibits an intermediate decrease in end bridging when compared to wildtype and *Δmre11* strains. Possibly, interaction between Sae2 and the Rad50S-mutant complex is impaired by the mutation. Since SSA deficiencies in *rad50S* strains were suppressed by overexpression of wildtype Sae2, end bridging in *rad50S* strains might also be rescued with increased expression of Sae2 protein.

HYPOTHESIS AND GOALS

The MRX complex in *S. cerevisiae* is implicated in DNA double-strand break repair during mitotic and meiotic growth and in the maintenance of genomic integrity. The Sae2 protein, along with the MRX complex, has been demonstrated to be required for the repair of specific DNA breaks, hairpin structures in vegetative cells and Spo11-induced DSBs during meiosis (Lobachev et al., 2002; McKee and Kleckner, 1997; Prinz et al., 1997). It has been suggested that the Sae2 protein may alter the MRX complex nuclease activity to remove hairpin structures and Spo11 adducts from DSB ends,

although the exact mechanism has not been explained. However, *Δsae2* strains exhibit intermediate sensitivity to MMS and other DNA damaging agents suggesting that other nucleases can compensate for Sae2 loss and that there are other functions that Sae2 participates in. Lack of the Sae2 protein decreases 5' end resection, single-strand annealing, and DNA end-bridging when compared to wildtype strains (Clerici et al., 2005b). Also, the DNA damage checkpoint persists much longer in cells lacking Sae2 (Clerici et al., 2004). This suggests that Sae2 functions not only in processing of specific DSBs but also plays a part in SSA possibly by ensuring association of broken DNA ends.

Characterizing the nuclease activity of the MRN(X) complex in DNA repair has been the foremost goal of this project. Since in vivo results suggest that the Sae2 protein is involved in regulating MRX nuclease activity, we have pursued biochemical characterization of the Sae2 protein also. There are no known homologs outside of fungi for the Sae2 protein and no obvious motifs to suggest the function(s) of the Sae2 protein. We expressed recombinant Sae2 protein as well as recombinant MRX complex to investigate the effects of Sae2 on MRX nuclease activity on various DNA substrates. Mutant Sae2 proteins were also expressed to investigate the function of domains within the protein. We biochemically characterized the Sae2 protein itself and in combination with the MRX complex, and found that the complexes exhibit functional cooperatively on some DNA substrates in vitro, in agreement with in vivo studies.

CHAPTER 2: MATERIALS AND METHODS

PLASMID CONSTRUCTION

Yeast Complementation Constructs

The *SAE2* gene was PCR amplified using primers TP775 (5'-ATGGATCCGT GACTGGTGAAGAAAATGTGTATC-3') and TP776 (5'-ATACTAGTTAAACATCT AGCATATATCTGCAATAATTTATC-3') with 5' *BamH*I and 3' *Spe*I restriction sites, respectively, from wildtype *S. cerevisiae* strain W303α (Thomas and Rothstein, 1989). The *SAE2* PCR product was introduced into pRS316 (Sikorski and Hieter, 1989) at *BamH*I and *Spe*I sites. Calmodulin Binding Protein (CBP) tag was PCR amplified from pBS1479 (Rigaut et al., 1999) using primers TP773 (5'-ATACTAGTGAAAAGAGAA GATGGAAAAAGAATTTC-3') and TP939 (5'-GCGGCCGCTCAGCTAGCAGT AGTTGGAATATCATAATC-3') was inserted into *Spe*I/*Not*I sites to yield *SAE2* C-terminally tagged with CBP in pTP690. The *SAE2* promoter region that included approximately 400bp of the *SAE2* gene was PCR amplified using primer TP1146 (5'-CGAGGTTACT TTAATAGTATATCTGA GACC-3') and TP1147 (5'-CGTGG ATTACAGTATCAG AGCAATCTTCC-3'), also from W303α. pTP712 was constructed by ligating a *Xho*I/*Afl*II fragment into pTP690 digested with *Sal*I/*Afl*II. pHIS3promMBP-SAE2 (gift from A. Rattray) contained *SAE2* with a Maltose Binding Protein (MBP)

fused at the N-terminal end, under the control of the constitutive HIS3 promoter from *S. cerevisiae*.

Mutants of the *SAE2* gene in pTP712 were constructed by using the Quikchange mutagenesis kit (Stragagene), cloning or gap repair. Quikchange mutagenesis using TP1288 (5'-CAAAATCCCCCCCAGATTTTGGAAGACTGG ATTTTC-3')/TP1289 (5'-GAAAATCCAGTCTTCCAAAATCTGGGGGGGATTTT G-3') primers yielded *sae2(G270D)* in pTP805. To mutate S73D, T90D, S249D, S279D and S289D sites, primers TP1104 (5'-ATGCTCCTCAACAATCCGATCA GACGTCTGCGGGGCCAGG-3')/TP1105 (5'-CCTGGCCCCGCAGACGTCTGA TCGGATTGTTGAGGAGCAT-3'), TP1106 (5'-GATTCTGAAGATTTCATCCTT GATCAGTTTGATGAGGACATAAAG-3')/ TP1107 (5'-CTTTATGTCCTCATCAA ACTGATCAAGGATGAAATCTTCAGAATC-3'), TP1108 (5'-CATTGTCAGTAGT TATAGAAGATCAAAATTCGGACTACGAATTTG-3')/TP1109 (5'-CAAATTCGTA GTCCGAATTTTGATCTTC TATAACTACTGACAATG-3'),TP1110 (5'-GAAGA CTGGATTTTCCCTCCGATCAGGAAGGGAACGAGGAC-3')/TP1111 (5'-GTC CTCGTTCCCTTCCTGATCGGAGGGAAAATCCAGTCTTC-3'), and TP1112 (5'-GGAACGAGGACAAAAAGAAAGACCAGGAAATCATCAGAAG-3')/1113 (5'-CT TCTGATGATTTCCTGGTCTTTCTTTTTGTCCTCGTTCC-3'), respectively, were used to yield *sae2(5D)* yielding pTP1122. pTP898 *(sae2(5A))* was constructed by gap repair between pTP712 digested at *Nru*I and *BsiW*I and pML487 (gift M.P. Longhese). The *SAE2* gene was PCR amplified with primers TP1335(5'-GGA

TCCATGGTGACTGGTGAAGAAAATGTGTATC-3')/ TP1334(5'-ACTAGTCG ATTCTATAACTACTGACAATG-3'), deleting amino acids 251-345 from the final gene product. The *sae2(ΔC)* PCR product was cloned into pTP712 between the *Afl*II and *Not*I sites to yield pTP898. A *Sac*I site outside of the *SAE2* in pTP712 was abolished by Quikchange mutagenesis using TP1758 (5'-CACTATAGGGC GAATTGGTGCTCCACCGCGGTGGCG-3')/TP1759 (5'-CGCCACCGCGGTGG AGCACCAATTCGCCCTATAGTG-3'). Deletion of the fragment between the two remaining internal *Sac*I sites and religation yielded pTP1131 *(sae2(ΔN))*, in which 21-172 amino acid residues were deleted.

Protein Expression Constructs for E. coli

The wildtype Sae2 expression construct, pExp566.gck, was a gift from A. Rattray. The *SAE2* gene in this construct is under the control of a T7 promoter, and 6xhistidine and MBP are fused at the N-terminus. pTP952 *(sae2(G270D))* and pTP1095 *(sae2(5D))* was constructed by Quikchange mutagenesis similarly to pTP805 and pTP1122, respectively. Using primers TP1648 (5'-GCGAAAGA TCCACGTATTGCC-3') and TP1649 (5'-GCTAGCCTGCAGTCAGGATCCGCC CTGGAAGTACAGATTTTCGC-3'), part of the MBP gene was PCR amplified from pExp566.gck to include 5' *BamH*I-stop-*Pst*I-*Nhe*I 3' restriction sites at the end of the MBP gene. The MBP PCR product digested by *Nco*I and *Nhe*I was inserted into pExp56.gck at *Nco*I/*Nhe*I, deleting the *SAE2* gene to construct

pTP1071. *sae2(5A)* was PCR amplified from pML487 with primers TP1335 (5'-GGATCCAT GGTGACTGGTGAAGAAAATGTGTATC-3')/ TP1612 (5'-CTGCA GTTAACATCT AGCATATATCTGC-3') and inserted in pTP1071 at *BamH*I and *Pst*I sites. *sae2(ΔC)* was amplified from pExp566.gck with primers TP1335/ TP1360 (5'-GCGGCCGCCTACGATTCTATAACTACTGACAATG-3') digested with *Nru*I and *Not*I sites and inserted into pExp566.gck to yield pTP1088. To construct the *sae2(ΔN)* expression vector, pTP1031, two internal *Sac*I sites deleted the N-terminal region of the *SAE2* in pExp566.gck similarly to pTP1131.

Protein Expression Constructs for Baculovirus

Constructs to express yeast Mre11 and Rad50 were previously described in (Bhaskara et al., 2007). Mre11 contains a C-terminal 6xhisitidine tag, pTP404. pTP404 was modified by Quikchange mutagenesis using primers TP685 (5'-GATTTTAATTAC TACAGCTAATCATGTGGGTTAC-3')/TP686 (5'-GTAACCCACATGATTAGCTGT AGTAATTAAAATC-3') and TP585 (5'-TATTCGGCATATCAGGTAATCTTGTTGA TGCGTCGGGGGACTCAC-3')/ TP586 (5'-GTGAGTCCCCCGACGCATCAACAAGATTACCTGATATGCCG AATA-3') to make *mre11-D16A* and *mre11-3(H125N/ D126V),* respectively. Rad50 expression construct, pTP684, was modified by Qukchange mutagenesis using primers TP483 (5'-TTTGACTCCAATGATATGGAAACTATT GAATTTGG-3') /TP484 (5'-CCAAATTCAATAGTTTCCATATCATTGGAGTC AAA-3') to make

rad50-R20M. The *XRS2* gene was PCR amplified from cDNA (Research Genetics) using primers TP1127 (5'-GTCGACATGTGGGTAGTA CGATACCAGAATAC-3') and TP1180 (5'-GGTACCTCATTTATCGTCGTCGT CCTTGTAGTCGCCTCCTTTTCTTCTTTTGAACG-3') that included a C-terminal Flag tag. *XRS2*-Flag PCR product was cloned into pFastBac1 yielding pTP694. pTP694 was made into a bacmid as previous described (Bhaskara et al., 2007) to make pTP701.

Human Mre11/Rad50/Nbs1 expression constructs and purification were previously described (Paull and Gellert, 1998; Paull and Gellert, 1999).

PROTEIN EXPRESSION AND PURIFICATION

Protein Expression of HisMBP-Sae2 Protein

All Sae2 expression constructs were transformed into the ArcticExpress strain (Invitrogen) and grown according to the manufacturer's instructions. 25mL Luria Broth (LB) and 1mg/mL ampicillin cultures were grown overnight and used to inoculate 2L LB with no ampicillin according to manufacturer's instructions. The cultures were grown to 0.8-1 O.D. at 25-30°C. The temperature was reduced to 13°C for 1 hour prior to induction. Protein expression was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 hours. Cells were harvested and frozen in liquid nitrogen.

Purification of HisMBP-Sae2 Protein

All steps were performed at 0-4°C. All buffers contained 0.5% (v/v) Tween-20 except where mentioned. Cells were resuspended in 20mL A buffer (25mM Tris pH8.0,10% glycerol, 20mM β-mercaptoethanol (BME)), containing 100mM NaCl, no Tween-20, 5mg/mL lysozyme and 1mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 minutes. The lysate was sonicated 3 times for 20 seconds each and then ultracentrifuged at 100,000 X g for 1 hour. The soluble fraction was applied to a 10mL Amylose column (New England Biolabs, NEB) equilibrated in A buffer with 100mM NaCl. The resin was washed with 50mL of 100mM NaCl A buffer and then 50mL of 50mM NaCl A buffer. The protein was eluted with 50mL of 50mM NaCl A buffer plus 10mM maltose. The protein fraction was collected and applied to a 10mL SP-Sepharose column (Amersham). The resin was washed with 50mL of 50mM NaCl A buffer and eluted with 50mL 600mM NaCl A buffer. The protein fraction was applied to Ni-NTA (Qiagen) resin washed with 50mL of 500mM KCl NiA buffer (50mM KH_2PO_4 pH 7.0, 10% glycerol, 20mM BME), 2 times with 50mL of 500mM LiCl, and 2 times with 50mL of 50mM KCl NiA buffer. The protein was eluted with 30mL of 50mM NaCl A buffer including 250mM imidazole pH 8.0. The protein fraction was loaded onto a SP-Sepharose HiTrap column (Amersham) equilibrated into 50mM NaCl A buffer. The protein was eluted with 600mM NaCl A buffer and peak fractions were collected and diluted with A buffer containing no salt to a final

concentration of approximately 50mM NaCl. The protein elution was loaded onto a second SP-Sepharose HiTrap column and eluted similar to the previous column. The peak fraction was loaded onto a Superdex-200 (Amersham) gel filtration column equilibrated into 100mM NaCl A buffer without Tween-20 but containing 1mM 1,4-dithiothreitol (DTT). All fractions after the exclusion volume were tested for nuclease activity. Fractions #24-27 were used for all activity assays.

Protein Expression and Purification of the MRX complex

Mre11/Rad50 protein complex: The wildtype Mre11 and Rad50 expression constructs were expressed from pTP404 and pTP684 and purified as previously described (Bhaskara et al., 2007). M(D16A)R and Mre11-3/R mutant complexes were coexpressed with baculovirus made from plasmids pTP552/pTP684 and pTP484/pTP684, respectiviely. For purification, cell lysate was applied to Ni-NTA resin, 1mL Hi-Trap Q-Sepharose, and Superose 6 HR10/30 gel filtration column. All MR complexes were purified as previously described (Bhaskara et al., 2007).

Mre11/Rad50/Xrs2 protein complex: The wildtype Mre11, Rad50 and Xrs2 proteins were expressed from baculovirus made from pTP404, pTP684 and pTP701 in Sf21 insect cells similarly as MR complexes. Approximately 7 grams of cells were lysed in 40mL A buffer containing 500mM NaCl and 1mM PMSF, sonicated and then ultracentrifuged at 100,000 X g for 1 hour. The soluble

fraction was treated with ammonium sulfate and the precipitate was collected after two steps 10% and 45% ammonium sulfate and centrifuged at 48,000 X g for 45 minutes each. The precipitate was resupended in zero salt A buffer to lower the conductivity to the equivalent of approximately 500mM NaCl. The protein solution was applied to 10mL of Ni-NTA resin and was washed with 50mL of 500mM KCl NiA buffer, 50mL of 50mL KCl NiA buffer, 50mL of 10% 50mM KCl NiB buffer (50mM KH_2PO_4 pH 7.0, 10% glycerol, 250mM imidazole, 20mM BME). The protein was eluted with 30mL of 50mM KCl NiB buffer. The protein fraction was then applied to a 1mL HiTrap Heparin column (G.E.) equilibrated in 100mM NaCl A buffer and eluted with 600mM NaCl A buffer. The peak fractions were incubated with 1mL anti-Flag M2 agarose resin (Sigma) at 4°C for 1 hour. The resin was washed with 10mL of 100mL NaCl A buffer. The protein eluted with 2mL each of 1M NaCl A buffer, 2M NaCl A buffer, 4M NaCl A buffer and high pH buffer (25mM K_2HPO_4 pH 12.5, 100mM NaCl, 10% glycerol). All fractions were dialyzed separately into 100mM NaCl A buffer with 1mM DTT. All fractions were tested for exonuclease activity. 2M, 4M and high pH fractions were used in the following assays.

Topoisomerase IIα Expression and Purification

pYepWob6 (gift from J. Wang) contains human topoisomerase IIα under a galatose-inducible promoter. Topoisomerase IIα was expressed in BJ5464α

(*ura3-52 trp1, leu2leu2-Δ1 his3-Δ200 pep::HIS3 prb-Δ16R can1 GAL*) (gift from C. Chan). Topoisomerase IIα was overexpressed and purified as previously described (Worland and Wang, 1989). For future experiments mutant human topoisomerase II (S763W) will be used. pGAL1-htop2-S763W (gift from J. Nitiss) will be used as before to overexpress topoisomerase II (S763W).

YEAST STRAINS

All strains used in this study are listed in Table 2.1. All strains for the MMS survival assay are isogenic to KSC1516 (*MATa-inc ADH4cs::HIS2 ade1 his2 leu2 trp1 ura3*)(gift from K. Sugimoto) . The *SAE2* gene was deleted using the *sae2::kanMX* cassette from *Δsae2* in BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*, Yeast Deletion set from Invitrogen). Strains including inverted *Alu* repeats are all isogenic to ALE94 (*MATa ade5-1 his7-2 leu2-3 112::p305L3 (LEU2) trp1-289 ura3-Δ lys::AluIR*) (gift from K. Lobachev). Strains used for m-AMSA survival assays are isogenic to JN362a (*MATa ISE2 ura3-52 leu2 trp1 his7 ade1-2*) (gift from J. Nitiss). *Δsae2* was constructed as for TP2162 and TP2464. The *RAD52* gene was deleted using the *rad52::LEU2* cassette from

Table 2.1

Yeast Strains used in this study

Strain	Geneotype	Strain construction
Strains for Table 3.1[a]		
TP2464	*sae2::kanMX*	
TP2478	*sae2::kanMX* (pRS316)	TP2464 + pRS316
TP2479	*sae2::kanMX* (pTP712; wildtype *SAE2-CBP*)	TP2464 + pTP712
TP2484	*sae2::kanMX* (pTP805; *sae2(G270D)-CBP*)	TP2464 + pTP805
TP2482	*sae2::kanMX* (pTP886; *sae2(5A)-CBP*)	TP2464 + pTP886
TP2483	*sae2::kanMX* (pTP898; *sae2(ΔC)-CBP*)	TP2464 + pTP898
TP2804	*sae2::kanMX* (pTP1122; *sae2(5D)-CBP*)	TP2464 + pTP1122
TP2848	*sae2::kanMX* (pTP1131; *sae2(ΔN)-CBP*)	TP2464 + pTP1131
TP2517	*sae2::kanMX* (pHISpromMBP-Sae2; wildtype *MBP-SAE2*)	TP2464 + pHISpromMBP-Sae2[d]
Strains for Figure 3.12[b]		
TP2162	*sae2::kanMX*	
TP2207	Wild-type *SAE2*	KSC1516 + pRS316
TP2208	*sae2::kanMX* (pTP712; wildtype *SAE2-CBP*)	TP2162 + pTP712
TP2210	*sae2::kanMX* (pTP805; *sae2(G270D)-CBP*)	TP2162 + pTP805
TP2430	*sae2::kanMX* (pTP886; *sae2(5A)-CBP*)	TP2162 + pTP886
TP2451	*sae2::kanMX* (pTP898; *sae2(ΔC)-CBP*)	TP2162 + pTP898
TP2802	*sae2::kanMX* (pTP1122; *sae2(5D)-CBP*)	TP2162 + pTP1122
TP2847	*sae2::kanMX* (pTP1131; *sae2(ΔN)-CBP*)	TP2162 + pTP1131
TP2348	*sae2::kanMX* (pHISpromMBP-Sae2; wildtype *MBP-SAE2*)	TP2162 + pHISpromMBP-Sae2
Strains for Figure 4.4[c]		
JN362a	Wild-type *SAE2* and *RAD52*	
TP2820	*sae2::kanMX*	
TP2821	*sae2::kanMX rad52::LEU2*	

[a] Strains isogenic to ALE94 (*MATa ade5-1 his7-2 leu2-3 112::p305L3(LEU2) trp1-289 ura3-D lys2::AluIR*) (Lobachev et al., 2000)

[b] Strains isogenic to KSC1516 (*MATa-inc ADH4cs::HIS2 ade1 his2 leu2 trp1 ura3*) (Nakada et al., 2004)

[c] Strains isogenic to JN362a (*MATa ISE2 ura3-52 leu2 trp1 his7 ade1-2*) (constructed by J. Nitiss)

[d] pHISpromMBP-Sae2 gift from A. Rattray

JN394 (*MATa rad52::LEU2 ISE2 ura3-52 leu2 trp1 his7 ade1-2*) (gift from J. Nitiss).

DNA BINDING

The DNA substrate was prepared by PCR amplification of a 250bp fragment in the [α-^{32}P] dATP. The PCR product was separated on a 1% agarose gel and extracted with the Gel Extraction kit (Qiagen). Gel mobility shift assays were performed in a volume of 28μL and 20μL for Sae2 only and Sae2 plus MRX assays, respectively. The final reaction concentrations were 25mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0 (MOPS), 2mM DTT, 10mM ethylenediaminetetraacetic acid (EDTA),100mM NaCl. The reactions were incubated on ice for 20 minutes with protein concentrations as indicated. The gel mobility shift assay with the Sae2 proteins only was resolved in 8% 37.5:1 acrylamide/ bisacryalmide (EMD) native gel, and the DNA binding assays with both Sae2 and MRX complexes were resolved in 0.7% agarose 1/2X 89mM Tris-Boric Acid 2mM EDTA ph 8.0 (TBE) gel. All DNA binding assays were analyzed by phosphorimager (BioRad or Molecular Dynamics).

OLIGONUCLEOTIDE SUBSTRATE PREPARATION

All 5' [^{32}P] labeled substrates were labeled with T4 polynucleotide kinase (PNK) (NEB). TP1442 (5'-GACAAGCGTACAGGTAATGCTCTGTACGCTTGT CGTCGATCTGG-3') was labeled at the 3' end with [^{32}P] cordecypin using

terminal deoxytransferase (TdT) (Boehringer). To anneal duplex substrates, the labeled strand and cold complement were boiled and slowly cooled in 100mM NaCl and 10mM Tris, pH 8.0 and 1mM EDTA (TE). Hairpin substrates were boiled and quickly cooled to 4°C with no salt.

Internally labeled hairpins TP1729 (5'-GTCGATCTGGGCATCTGTAA TGTGGCTGGAAGTAGGAGCG-3')/TP1492 (5'-CCAGATCGACGACAAGCGT ACAGGTAATGCTCTGTACGCTTGTC-3') and TP1589 (5'-GTCGATCTGGA GGGCGTACCAGTAGCTACTGGTAC-3')/TP1492 were constructed by 5' end labeling with [^{32}P] and ligating the two parts together overnight at 16°C with T4 ligase (NEB). The hairpins were lyophilized, resupended in formamide loading buffer, and separated on a 20% denaturing polyacrylamide sequencing gel. The appropriate band was cut out and the DNA was extracted with 1mL TE by rotating at 42°C overnight. The DNA solution was purified by phenol:chroloform and chloroform extractions. The DNA was precipitated from the aqueous fraction with 2 volumes of 100% EtOH and 0.1 volume of 3M NaOAc. After precipitation the DNA was washed with 70% EtOH, lyophilized and resuspended in TE and annealed.

NUCLEASE ASSAYS

Exonuclease Assays: Reactions were preformed in 10μL with 25mM MOPS, 2mM DTT, 50mM NaCl, 1mM $MnCl_2$ or 5mM $MgCl_2$ as indicated and

50mM NaCl. The exonuclease assays were performed with oligonucleotide substrate TP74 annealed to TP124 (Paull and Gellert, 2000) at 37°C for 20 minutes.

Endonuclease Hairpin Assay: Reactions with TP1442 were performed in 10μL total with 25mM MOPS, 2mM DTT, 50mM NaCl, 1mM $MnCl_2$ and 0.5mM ATP for 45 minutes at 37°C. Assays with internally labeled hairpins, TP1729/TP1492 and TP1589/TP1492, were performed with 5mM $MgCl_2$ instead of $MnCl_2$ and no ATP. Complementary DNA strands to ssDNA on TP1729/TP1492 were TP1754 (5'-TCCTACTTCCAGCCACATTACA-3') and TP1755 (5'-TCCTACTTCCAGCCAC-3'). Oligonucleotide substrates analogous to TP1729/TP1492 was TP1736 (5'-GTCGATCTGGGCATCTGTAATGTGGCTG GAAGTAGGAGCGCTGTACGCTTGTC-3')/TP1735 (5'-CCAGATCGACGACAA GCGTACAG-3'). All assays with control substrates for the internally labeled hairpins were performed similarly to the conditions for the corresponding hairpin assay. Assays with TP1589/TP1492 were performed in a total of 10μL in 25mM MOPS, 2mM DTT, 50mM NaCl, and 1mM $MnCl_2$ with MRX or MR complexes for 20 minutes at 37°C. $MgCl_2$ was added to a final concentration of 5mM along with the Sae2 protein and incubated for 30 more minutes.

All nuclease reactions were resolved in 20% denaturing polyacyralmide gels (20% acrylamide, 7.5 urea, 1X TBE), which were analyzed by phosphorimager (BioRad or Molecular Dynamics).

MMS SURVIVAL ASSAYS

Survival assays were performed as previously described (Moncalian et al., 2004). Briefly, each experiment was performed in triplicate from single colonies grown in synethic medium lacking uracil with 2% glucose and subcultured to grow 100mL cultures to early log-phase (between 0.7 and 1.0 A_{600} unit/ml). These cultures were harvested and 10 A_{600} units were resuspended in synthetic medium with or without MMS at indicated concentrations. Cultures were grown for five hours. Cells from treated and untreated cultures were plated in serial dilutions on plates containing synthetic medium lacking uracil. The percentage survival was calculated by dividing the number of viable cells from the treated cultures by the number of viable cells from the untreated cultures. The average survival was calculated for each point with standard deviations.

RECOMBINATION RATE BETWEEN INVERTED REPEATS

Rates of recombination induced by inverted *Alu* repeats were determined as previously described (Lobachev et al., 2002). Briefly, the *SAE2* gene was deleted using the *sae2::kanMX* cassette from *Δsae2* in BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*, Yeast Deletion set from Invitrogen) in the ALE94 strain (gift from K. Lobachev). *SAE2* wildtype and mutant genes were transformed into this strain on *CEN ARS URA3*-containing plasmids (derivatives of pRS316). Subsequent strains were grown in triplicate from single colonies in

synthetic medium lacking uracil, grown to log-phase and subcultured starting at 0.1 A_{600}. Each culture was washed three times and 0.1-2 A_{600} was plated on synthetic medium lacking lysine. To account for differences in growth of each strain, the number of viable cells/mL were calculated by plating cells on rich medium. The rate of recombination for every 10^7 cells was calculated for each strain in triplicate and standard deviation is as shown.

TOPOISOMERASE II CLEAVABLE COMPLEX ASSAYS

Plasmid Cleavable Complex

Supercoiled pUC18 was separated in 0.7% aragose (1X TBE) gel from open-circular and linear species. A sample lane was stained with ethidium bromide (EtBr) and the supercoiled species was measured from well. Supercoilded (sc) pUC18 was electroeluted into TE without EtBr staining. Topoisomerase II was incubated with 50ng of supercoiled pUC18 with 25mM MOPS, 2mM DTT, 5mM $MnCl_2$, 1mM ATP and 400mM etoposide, as indicated, for 30 minutes at 37°C. Wildtype MRN complex was added for another 30 minutes at 37°C. Mung bean and proteinase K were added last for 15 minutes. Reactions were stopped with 1% Sodium dodecyl sulphate (SDS) and 0.5mM EDTA. Products were resolved in a 1% agarose gel in 1X TBE containing 0.1% SDS.

Oligonucleotide Cleavable Complex

Internally labeled oligonucleotide substrates were prepared as described above for DNA hairpin substrate preparation. TP692 (5'-AATGATT*T*A*C*T*T-3') contains five phosphorothioate bonds (asteriks) at the 3' end. Previous results demonstrated that phosphorothioate bonds block human MRN 3'→5' exonuclease activity (unpublished data, T.T. Paull). TP692 was 5' [^{32}P] labeled and ligated to TP770 (5'-CTGCAGGGTTTTTGTTCCGAGCTTAGCACTGTG TAAGACAGGCCGATGTGAGGATGACGATGAGCGCATTGTTAGATT-3') with complementary strand TP771 (5'-AAGTAAATCATTAATCTAACAATGCGCTCA TCGTCATCCTCACATCGGCCTGTCTTACACAGTGCTAAGCTCGGAACAAAA ACCC*T*G*C*A*G-3'). TP692+TP770/TP771 was used in the SDS-PAGE assay (Figure 4.2 C). TP692 was also labeled and ligated to TP691 (5'-AGGATGAC GATGAGCGCATTGTTAGATT-3') with complentary strand TP694 (5'-AAGTA AATCATTAATCTAACAATGCGCTCATCGT C*A*T*C*C*T-3'). TP692+TP691/TP694 was used in Figure 4.2B and resolved in 20% denaturing poyacryalmide gels. TP694 was also 5' end labeled and annealed to TP695 ('5-AGGATGACGATGAGCGCATTGTTAGATTAATGATT*T*A*C*T*-3') and used in Figure 4.3 and also resolved in 20% denaturing poyacryalmide gels. Conditions for Topoisomerase IIα and human MRN were similar to the plasmid cleavable complex assays.

m-AMSA Survival Assays

Wildtype, *Δsae2* and *Δsae2Δrad52* strains were grown to confluency from single colonies in 5mL yeast extract-peptone-dextrose medium and adenine medium (YPAD) . Cultures were subcultured into 50mL of YPAD and grown to early log phase (between 0.7 and 1.0 A_{600} unit/ml). These cultures were harvested and 10 A_{600} units were resuspended in YPAD with or without m-AMSA at indicated concentrations and grown for 24 hours. Cells from treated and untreated cultures were plated in serial dilutions on YPAD plates. Calculations were conducted similar to the MMS survival curves.

CHAPTER 3: THE EFFECTS OF THE SAE2 PROTEIN ON MRX NUCLEASE ACTIVITY

INTRODUCTION

In *S. cerevisiae*, the repair of DNA double-strand breaks (DSBs) requires several factors including the Mre11/Rad50/Xrs2 (MRX) complex and Sae2. The MRX complex has been studied extensively in vivo in budding yeast and has been found to play a critical role in the repair of DSBs through both homologous and nonhomologous pathways (Krogh and Symington, 2004). The Mre11 and Rad50 components of the MRX complex are conserved throughout all organisms, while Xrs2 and its functional homolog Nbs1 in mammals are found only in eukaryotes. However, the role of Sae2 is still not clearly understood and homologs have not yet been identified in other organisms. It has been demonstrated in vivo that Sae2 functions with MRX in DNA hairpin processing (Lobachev et al., 2002; Rattray et al., 2001) and in the removal of Spo11 conjugates during meiosis (Alani et al., 1990; Keeney et al., 1997).

To determine the specific role of Sae2 in DSB repair we expressed recombinant Sae2 in *E. coli* and performed in vitro assays on model DNA oligonucleotide substrates. Unexpectedly, we found that Sae2 itself functions as an endonuclease in the presence and absence of MRX. Sae2 independently exhibits ssDNA endonuclease activity and sequence-nonspecific double-strand

DNA binding. Consistent with the in vivo data, we also found that the MRX complex stimulates Sae2 to cut DNA hairpin structures. Sae2 has not previously been studied in vitro so the assays described here serve to characterize Sae2 biochemical functions.

EXPRESSION AND PURIFICATION OF RECOMBINANT SAE2

The *SAE2* gene was PCR amplified from yeast genomic DNA and was initially cloned with a C-terminal Calmodulin-Binding-Protein (CBP) tag for expression in yeast under the control of the inducible GAL1/10 promoter. However, preliminary experiments indicated a very low level of protein expression using this system. Another group had observed more reasonable levels of expression using an *E. coli* system in which Sae2 was expressed as a Maltose-Binding-Protein (MBP) fusion (Alison Rattray, personal communication). This construct expressed recombinant Sae2 under the control of a T7 promoter (gift from A. Rattray) and produced protein tagged with 6xhistidine and MBP on the N-terminal end. A purification strategy (described in Chapter 2) was developed using affinity (Nickel-NTA and amylose resins), ion-exchange (SP-Sepharose), and gel filtration (Superdex 200) chromatography. The purification strategy yielded recombinant Sae2 at nearly 100% homogeneity (Figure 3.3B),

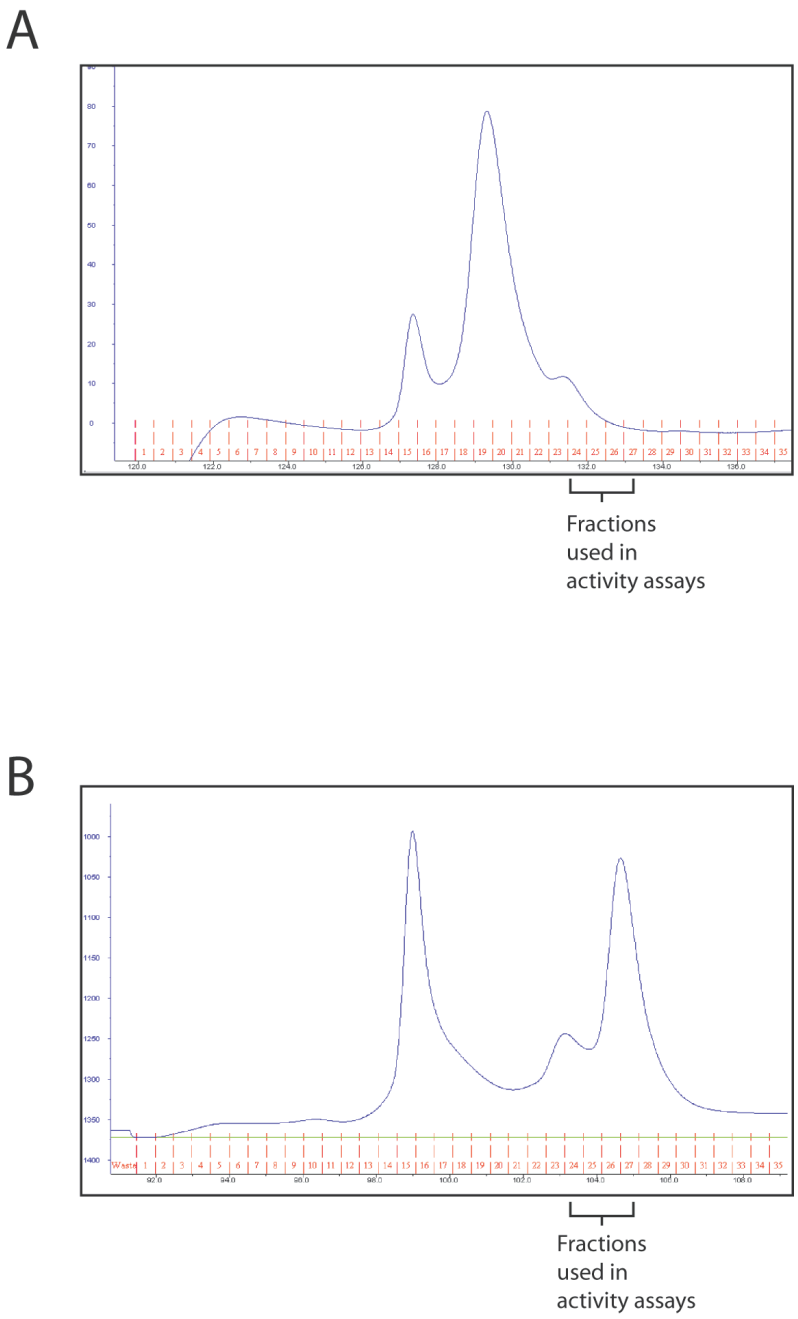

Figure 3.1 Recombinant wildtype and mutant Sae2 proteins form multimeric complexes. A) The UV absorbance curve of the recombinant wildtype Sae2 protein passed through a Superdex-200 gel filtration column. B) The UV absorbance curve of the Sae2(ΔN) mutant protein passed through the Superdex-200 column. Fractions #24-27 were used in activity assays.

although three distinct multimeric forms were observed during the gel filtration step of purification (Figure 3.1A). Preliminary sedimentation results suggest that mostly aggregated Sae2 protein is found in the first and second significant peaks, fraction #16 and #20 (data not shown). Further studies are currently underway to determine the multimeric state of the Sae2 protein in vivo and in vitro.

SAE2 MUTANTS

We created several mutants of Sae2 based on sequence conservation, on the results of a genetic screen performed by our collaborator A. Rattray, and on earlier studies in *S. cerevisiae* (Figure 3.2 and 3.3A). In order to determine essential domains necessary for protein-protein and protein-DNA interactions we created a C-terminal truncation removing 251-345 amino acids, Sae2(ΔC). In addition, internal *Sac*I restriction sites were used to remove amino acids 21-172 leaving the 20 amino acids of the N-terminus in frame with 173-345 at the C-terminus, Sae2(ΔN). Sae2(G270D) was discovered in a random mutational analysis screen for mutants deficient in the resolution of hairpins in vivo (Rattray et al., 2001) (A. Rattray, personal communication). Sae2(5A) and Sae2(5D) were based on the putative Tel1/Mec1 phosphorylation sites, S73, T90, S249, T279, and S289 identified by the Longhese group (Baroni et al., 2004). In the Sae2(5A) mutant each serine or threonine was changed to an alanine. Baroni et al showed that yeast strains expressing the Sae2(5A) mutant were nearly as

sensitive to methyl-methane-sulfonate (MMS) as *Δsae2* strains. In the Sae2(5D) mutant the five putative phosphorylation sites were changed to aspartates to mimic phosphorylation.

The *sae2(G270D), sae2(5D), sae2(5A), sae2(ΔN),* and *sae2(ΔC)* mutant ORFs were cloned into the same *E. coli* expression vector as the wildtype *SAE2* and purified as described above (Figure 3.3). Wildtype, Sae2(G270D), Sae2(5D) and Sae2(5A) mutants migrated on SDS-PAGE gels at the predicted size of 84 kDa and Sae2(ΔN) and Sae2(ΔC) were 67 kDa and 72 kDa, respectively (Figure 3.3). All mutants except for the Sae2(ΔN) showed identical profiles when passed through the gel filtration column. When the Sae2(ΔN) was analyzed by gel filtration, however, there was very little protein in the higher molecular weight fractions and the majority of the protein was in the third peak (Figure 3.1B). This suggests that full-length Sae2 proteins form multimers and that the Sae2(ΔN) mutant may lack a putative interaction domain. Interestingly, preliminary sedimentation data suggests that the Sae2(ΔN) peak fraction, #27, is a monomer (data not shown). Fractions in the range of the third peak were used for all of the experiments shown here, for wildtype as well as mutant proteins.

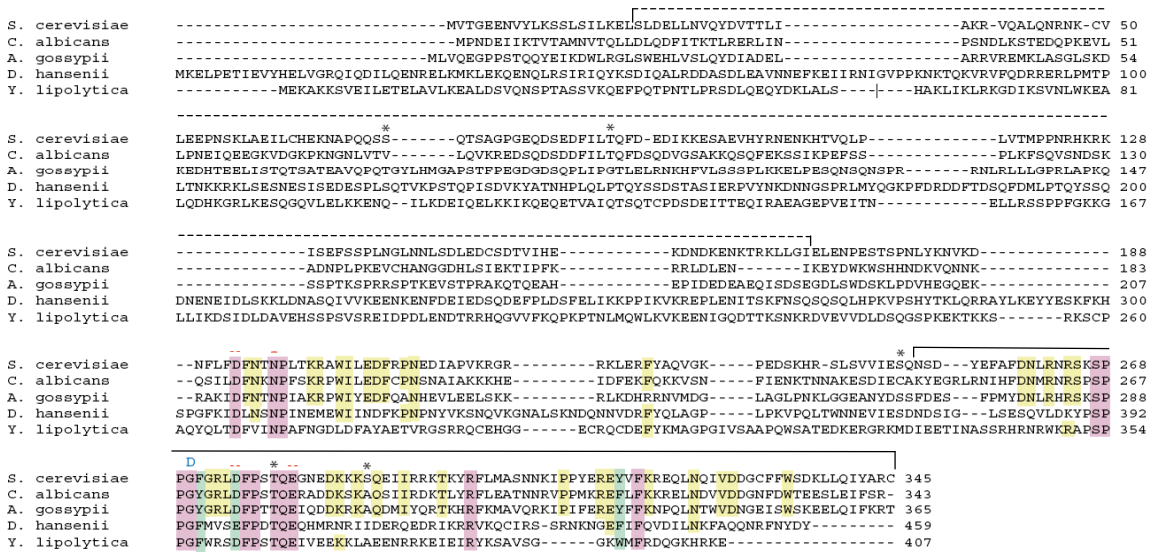

Figure 3.2 Sequence alignment of SAE2 homologs from fungi. S. cerevisiae, C. albicans, A. gosstypii, D. hansenii and *Y. lipolytica*. Invariant residues are highlighted in purple; residues with similarity across all 5 organisms are highlighted in green; residues with identity across at least 3 organisms are highlighted in yellow. Phosphorylation sites are indicated by the astericks. ΔN and ΔC deletions are indicated by a dotted and solid line, respectively.

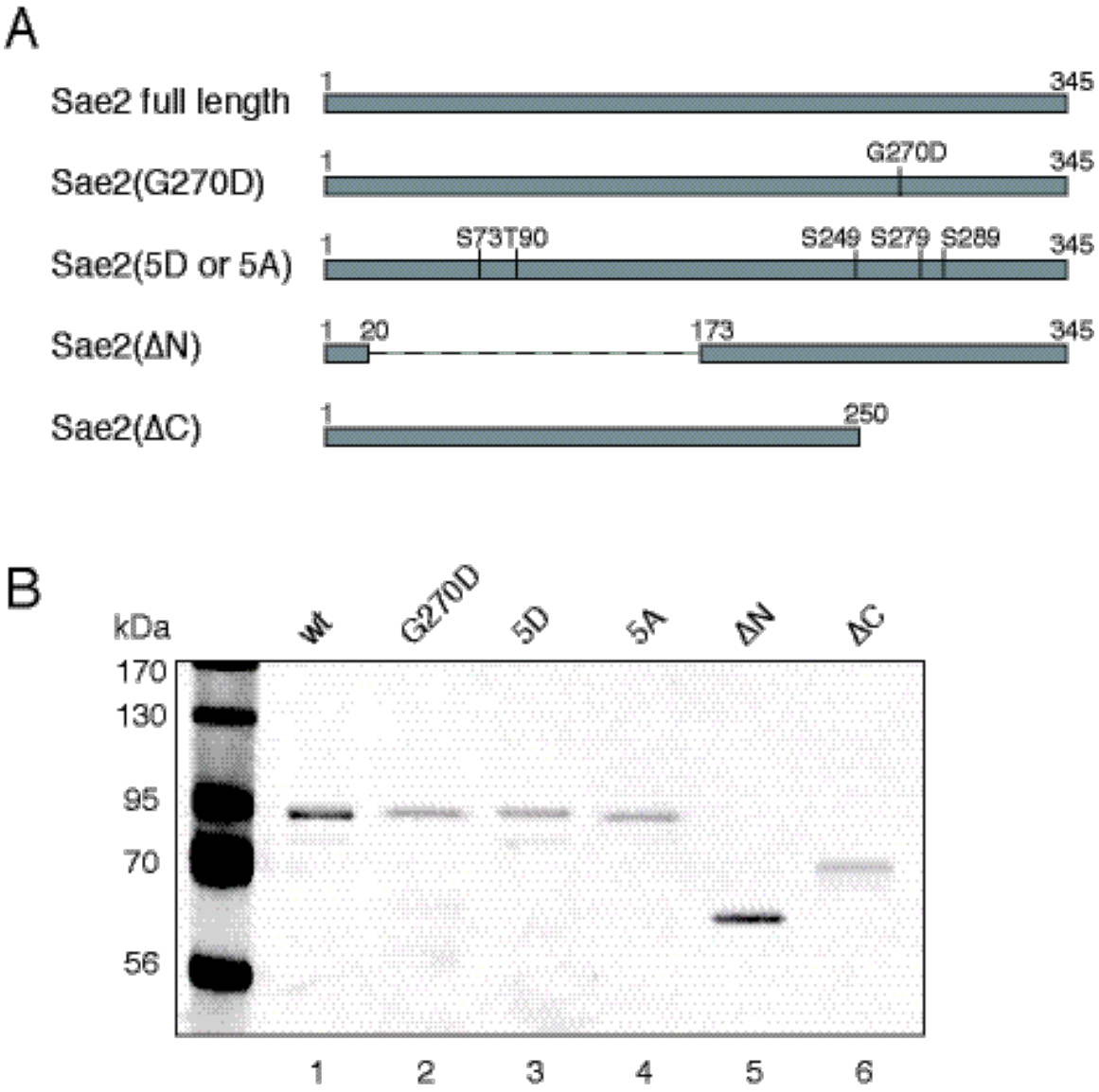

Figure 3.3 Sae2 mutant proteins. A) Schematic representation of wildtype Sae2, and mutant Sae2(G270D), Sae2(5D), Sae2(5A), Sae2(ΔN) and Sae2(ΔC) proteins. B) SDS-PAGE of purified Sae2 wildtype and mutant proteins, approximately 100ng total protein each.

SAE2 BINDS DNA

Sae2 has been shown to form foci at sites of DSBs, both in the presence and absence of MRX (Lisby et al., 2004). To test whether Sae2 is itself a DNA-binding protein we performed gel mobility shift assays, as shown in Figure 3.4A. Sae2 was incubated with a [^{32}P]-labeled 250bp double-stranded DNA substrate and analyzed in a 8% native acrylamide gel. We found that Sae2 forms a stable complex with DNA, evident by the mobility shift of the substrate (Figure 3.4A, lanes 2-3). The Sae2(5D), Sae2(5A) and Sae2(ΔC) mutants all showed DNA binding activity similar to wildtype Sae2 (Figure 3.4A, lanes 6-7, 8-9 and 12-13) while the Sae2(G270D) mutant showed very weak DNA binding and the Sae2(ΔC) mutant showed a complete loss of DNA-binding activity (Figure 3.4A, lanes 3-4 and 10-11).

Since in vivo data suggest that MRX and Sae2 cooperate in DNA end processing, we also investigated whether MRX affects Sae2 DNA binding. To do this recombinant MRX was expressed in a baculovirus system by coexpression (see Chapter 2 for details of purification), similar to the expression of human MRN (Paull and Gellert, 1998; Paull and Gellert, 1999). Purification using affinity (Nickel-NTA and anti-Flag agarose) and ion-exchange (Q Sepharose) chromatography yielded the complete MRX complex. The gel shifts with Sae2 and MRX proteins were performed with the same DNA substrate as in Figure 3.4A, although separated in an agarose gel because of the expected

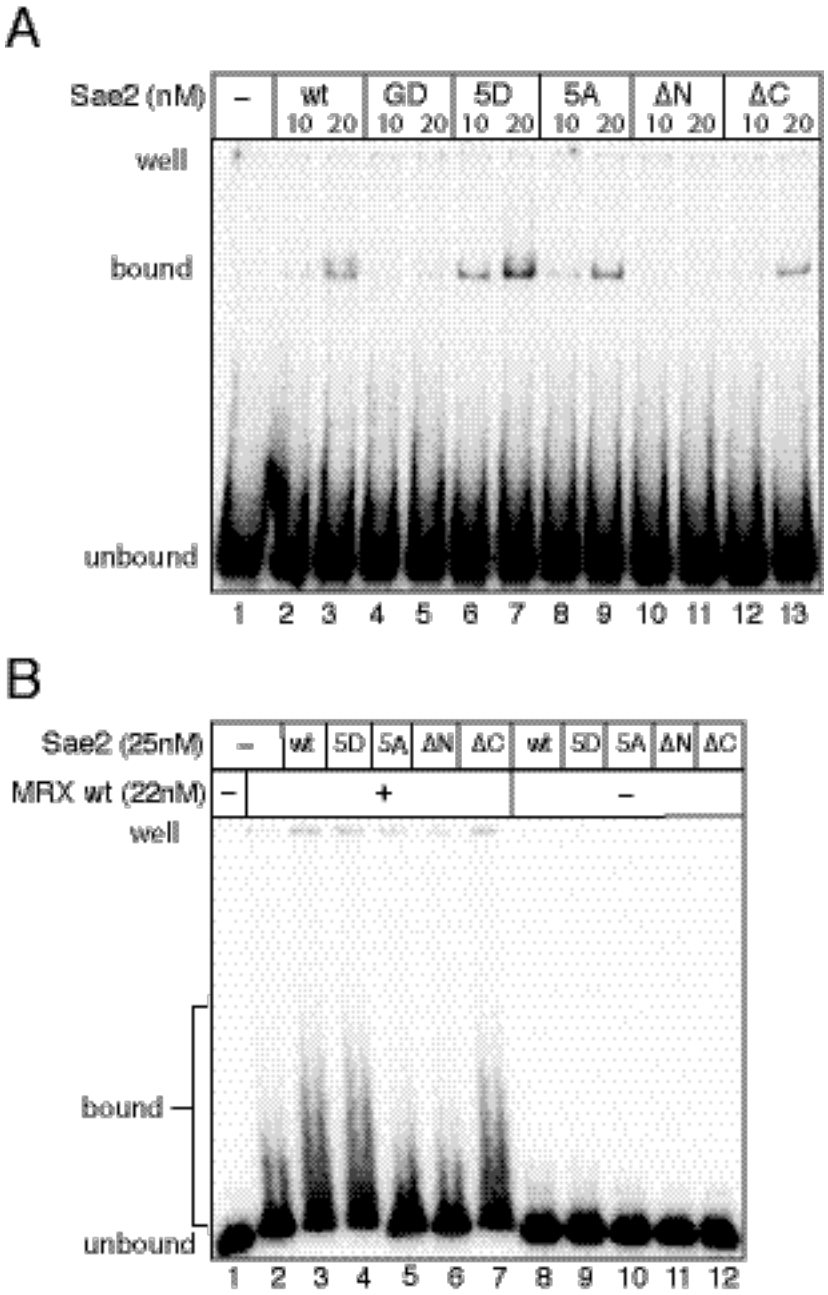

Figure 3.4 DNA binding of wildtype Sae2 and mutant proteins in the absence and presence of MRX. A) Increasing amounts of wildtype and mutant Sae2 proteins were incubated with a 2.5kb DNA substrate on ice. Sae2-DNA complexes were analyzed in 8% native polyacrylamide gel. B) The MRX complex was incubated with wildtype and mutant Sae2 proteins with a similar DNA substrate as in (A). The MRX-Sae2-DNA complexes were analyzed in 0.7% agarose gel.

large size of MRX-DNA complexes. MRX alone binds DNA but at these concentrations (22nM) does not form discrete higher order complexes (Figure 3.4B, lane 2). Interestingly, when combined with Sae2, MRX exhibits a greater mobility shift than with either protein alone (Figure 3.4B, lane 3 compared to lanes 2 and 8). Since MRX also can bind DNA, we asked whether Sae2 and MRX were binding the DNA together or independently. To do so, we tested MRX with several mutant Sae2 proteins. Wildtype Sae2, Sae2(5D) and Sae2(ΔC) are able to create a higher mobility shift when incubated with MRX (Figure 3.4B, lanes 4 and 7). Neither Sae2(5A) nor Sae2(ΔN) are able to increase the MRX shift (Figure 3.4B, lanes 5 and 6). Both Sae2(5A) and Sae2(ΔC) are able to bind DNA alone (Figure 3.4A, lanes 8-9 and 12-13). Nonetheless, the inability of Sae2(5A) to increase the MRX shift suggests that MRX and Sae2 may be interacting together on the DNA and not independently, and that the Sae2(5A) mutant is deficient in this interaction. We also tested for protein-protein interactions between MRX and Sae2 in the absence of DNA, but no significant interaction was observed (data not shown).

EFFECTS OF SAE2 ON MRX EXO AND ENDONUCLEASE ACTIVITIES

In vivo data suggests that Sae2 acts cooperatively with MRX in removing hairpin-capped DSBs (Lobachev et al., 2002), therefore we tested whether Sae2

affects MRX nuclease activity in vitro. Purified recombinant MRX complex exhibited 3'→5' exonuclease activity on a 3' recessed double strand DNA substrate in manganese but not in magnesium, as was reported previously for MRX (Trujillo and Sung, 2001) and similar to our previous findings with human MRN (Paull and Gellert, 1998; Paull and Gellert, 1999)(Figure 3.5A, lanes 2-5). The addition of the Sae2 protein stimulated the exonuclease activity of the MRX complex 2-4.5-fold when compared to MRX alone, and Sae2 did not exhibit any exonuclease activity by itself on this substrate (Figure 3.5A, lanes 6-8 and 9-10, respectively). To further characterize the activities of the MRX complex we purified the *rad50S* separation of function mutant, MR(R20M)X. Overexpression of Sae2 in a *rad50S* strain partially complemented defects in SSA observed in this strain (Clerici et al., 2005b). The MR(R20M)X mutant complex exhibited similar exonuclease activity compared to wildtype MRX (Figure 3.5B, lanes 4-5). Wildtype MR was also tested and showed similar exonuclease activity to the full complex, while the mutants in the nuclease domain, M(D16A)R (Furuse et al., 1998) and Mre11-3/R (Bressan et al., 1998), did not have exonuclease activity (Figure 3.5C, lanes 4-7), as previously reported (Lewis et al., 2004; Moreau et al., 1999).

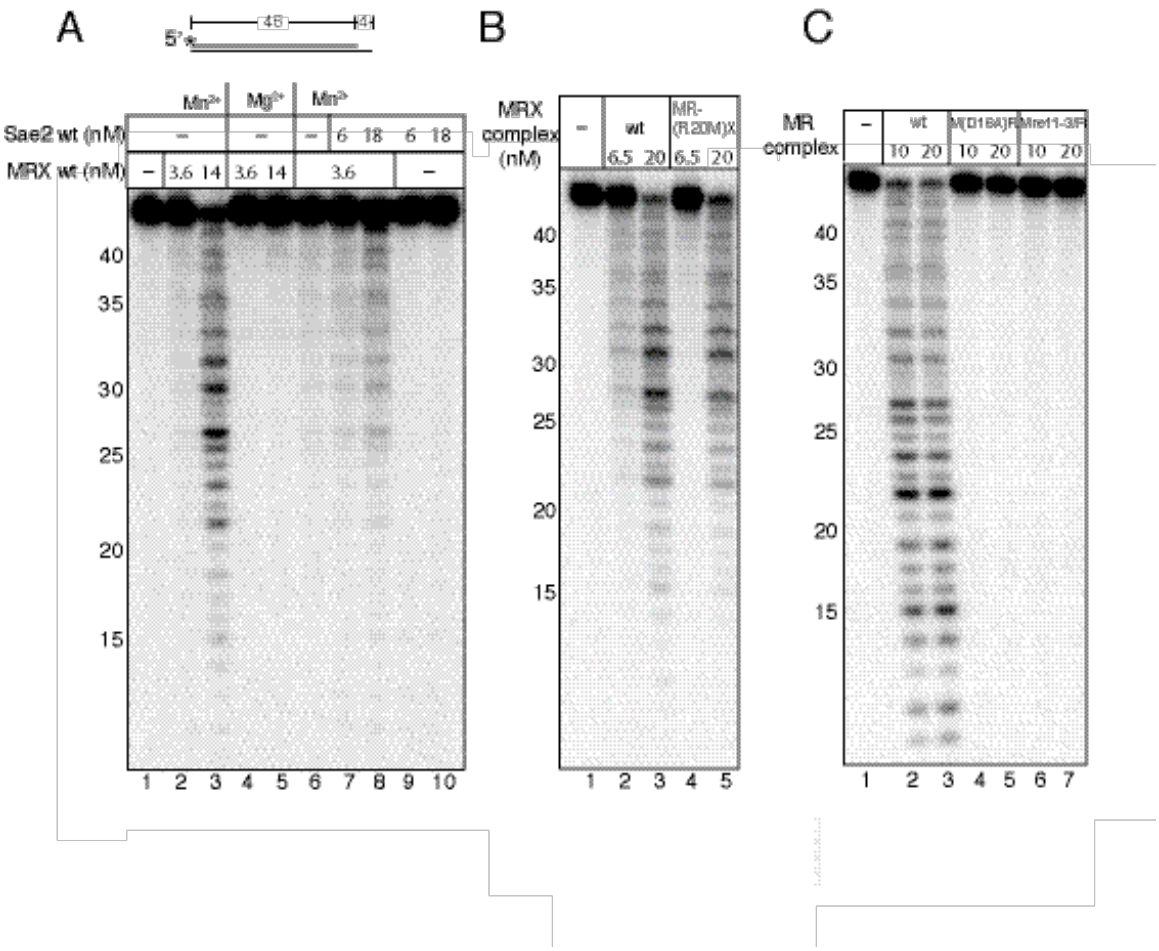

Figure 3.5 The Sae2 protein stimulates MRX 3'→5' exonuclease activity. A) Wildtype MRX exhibits exonuclease activity in manganese but not magnesium (lanes 2-5). The Sae2 protein stimulates MRX exonuclease activity 2-4.5 fold (lanes 6-8) but does not exhibit exonuclease activity itself (lanes 9-10). B) Mutant MR(R20M)X (lanes 4-5) complex exhibits similar exonuclease activity when compared to wildtype MRX (lanes 2-3). C) Both M(D16A)R and Mre11-3/R mutant complexes (lanes 4-7) do not exhibit exonuclease activity when compared to wildtype MR complex (lanes 2-3). Products were resolved in 20% denaturing polyacrylamide gels.

DNA HAIRPIN SPECIFIC ENDONUCLEOLYTIC ACTIVITY OF SAE2

Lobechev et al. demonstrated that MRX and Sae2 are required for the resolution of hairpin-capped DSBs at sites of cruciform formation in vivo (Lobachev et al., 2002). Though neither MRX nor Sae2 are required to cleave the base of the cruciform, deletion of *SAE2* or any of the MRX components resulted in the retention of the hairpin-capped DNA ends, ultimately leading to inappropriate gene amplification events. Considering this evidence, we investigated MRX and Sae2 nuclease activity on a DNA hairpin structure in vitro.

A previous study showed that recombinant yeast MR and MRX cut DNA hairpin structures at the tip (Trujillo et al., 2003; Trujillo and Sung, 2001), similar to previous results with human MRN (Paull and Gellert, 1999) and with *E. coli* SbcC/D (Connelly et al., 1998). We tested our MR and MRX (data not shown) complexes for hairpin endonuclease activity on a hairpin containing an 8nt loop, a 13-base pair stem and a 10-nucleotide 3' overhang and found that both forms of the complex exhibit relatively inefficient but detectable cutting of the hairpin oligonucleotide substrate at the tip (Figure 3.6, lanes 2 and 5). The addition of Sae2 did not affect the MR(X) hairpin cutting activity (Figure 3.6, lanes 3-4 and 6-7).

During the course of our experiments with MRX and Sae2 on various hairpin structures, we observed that Sae2 itself exhibited hairpin-cutting activity

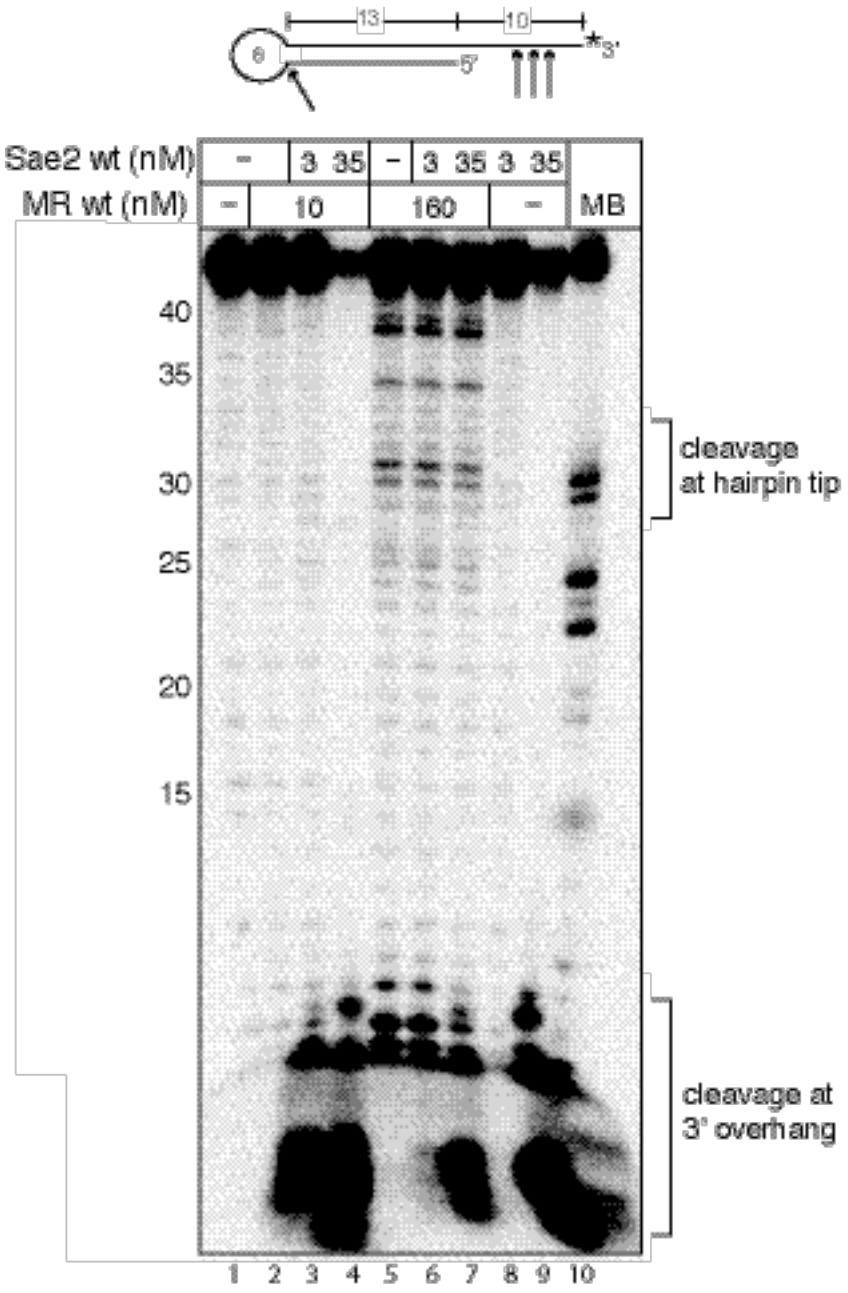

Figure 3.6 MR complex and Sae2 complexes exhibit endonuclease activity at different sites on a DNA hairpin structure. MR cleaves at a hairpin tip at 160nM but to 10mM in 1mM manganese and 0.5mM ATP. The Sae2 protein does not stimulate MR cleavage at the tip. However, Sae2 exhibits endonuclease activity at the ssDNA overhang. This activity is stimulated in the presence of MR (compare lanes 2, 3 and 8). MB; mung bean nuclease.

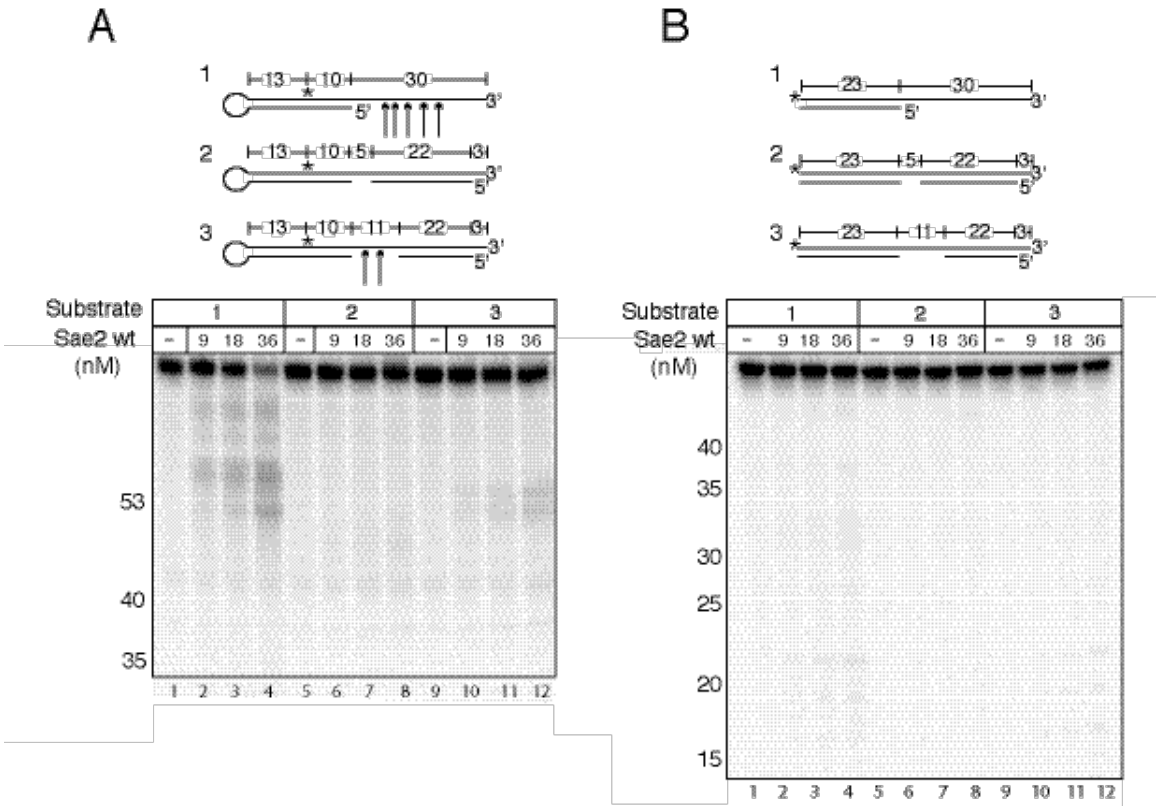

Figure 3.7 The Sae2 protein exhibits endonuclease activity. A) Sae2 exhibits endonuclease activity on ssDNA adjacent to DNA hairpin structures (lanes 2-4). Endonuclease activity was altered when complementary DNA strands were annealed to the 3' overhang but retained a 5-nucleotide gap (lanes 6-8) and an 11-nucleotide gap (lanes 10-12). B) Substrate 1, 2 and 3 are analogous to substrates 1, 2 and 3 in (A) except they lack the hairpin structure. The Sae2 protein exhibited little endonuclease activity on these substrates.

on some hairpin substrates. For instance, an internally [^{32}P]-labeled hairpin was generated with an 8-nucleotide loop and 3' overhangs, as shown in Figure 3.7A. Addition of recombinant Sae2 to this substrate resulted in the production of several products (Figure 3.7A, lanes 2-4). This activity was seen in both magnesium and manganese, and the assays shown here were performed in magnesium. The distribution of products suggested that Sae2 cut the hairpin not at the tip but at the single-strand DNA adjacent to the hairpin (Figure 3.7A, lanes 2-4). This activity can also be seen with the 3'-labeled hairpin shown in Figure 3.6. To test the hypothesis that Sae2 cuts single-stranded DNA adjacent to hairpin structures, variants of this substrate were generated that included complementary strands annealed to the overhang to block potential cut sites but retain a 5-nucleotide gap or an 11-nucleotide gap. When compared to the hairpin without a complementary strand, Sae2 was able to cut the variant including an 11-nucleotide gap but with 2.3-fold less efficiency than the substrate lacking a complementary strand (Figure 3.7A, lanes 10-12). With only a 5-nucleotide gap on the substrate, Sae2 was not able to generate any products (Figure 3.7A, lanes 6-8). These results indicated that Sae2 itself is an endonuclease that can cleave ssDNA at ends or in gaps.

To investigate the requirement for DNA hairpin substrates in Sae2 endonuclease activity, duplex DNA substrates were created that are identical to the hairpin variants but lacking the hairpin loops. Sae2 showed minor

endonuclease activity on the substrate containing the 3' overhang (Figure 3.7B, lanes 2-4) 10-fold lower than the equivalent hairpin-containing substrate. Sae2 also showed extremely low endonuclease activity on the substrates analogous to the hairpins with complementary strands (Figure 3.7B, lanes 6-8 and 10-12). The endonuclease activity of Sae2 on ssDNA and gaps therefore appears to be strongly stimulated by the presence of an adjacent hairpin structure.

HAIRPIN REMOVAL WITH SAE2 AND MRX

The ability of Sae2 to cleave DNA adjacent to hairpin structures suggested the possibility that MRX might stimulate Sae2 rather than Sae2 stimulating MRX activity. To test this hypothesis, an internally labeled hairpin with an 8-nucleotide loop and a 3' overhang (as shown in Figure 3.7A) was prepared and incubated with recombinant MRX and Sae2 in presence of magnesium ions, which supports Sae2 but not MRX endonuclease activity. In the presence of both complexes, a product was formed which migrated higher than the expected product from a hairpin cleavage at the tip, yet was clearly dependent on both complexes (Figure 3.8A, lanes 3-4). MRX and Sae2 acted cooperatively on this substrate in magnesium, indicating that Mre11 nuclease activity is not required for product formation. The stimulatory effect of MRX on Sae2 on this substrate was specific to hairpin structures, because no stimulation or cooperatively was observed with an identical DNA substrate that lacked the hairpin tip (Figure 3.8B, lanes 3-4).

Although Sae2 could cut the single-stranded overhang adjacent to the hairpin without MRX (Figure 3.7A) the concentrations of Sae2 used here in combination with MRX were 3-12-fold lower than the levels required for Sae2 endonuclease activity in the absence of MRX. Similarly, the levels of the MRX complex necessary for cooperative activity with Sae2 were 16-fold lower than the levels required for hairpin cleavage at the tip by MRX alone (Figure 3.6).

The Sae2 mutants described above were all tested in comparison to wildtype Sae2 with MRX on this hairpin substrate, as shown in Figure 3.8A. Sae2(5D) was the only mutant that exhibited cooperative endonuclease activity with MRX when tested with the hairpin substrate (Figure 3.9A, lane 5). The Sae2(G270D) and Sae2(ΔN) mutants were both completely deficient in hairpin endonuclease activity in this assay (Figure 3.9A, lanes 4 and 6), consistent with their lack of DNA-binding activity (Figure 3.4A). To characterize the contribution of individual components of the MRX complex for cooperative activity with Sae2 on the hairpin substrate, MR and MR(R20M)X were tested with Sae2. MR was able to act cooperatively with Sae2 to cut the hairpin substrate (data not shown) suggesting that Xrs2 is not required for this stimulation. In vivo data showed that yeast strains expressing the Rad50S mutant complex could be partially suppressed for single-strand annealing defects by overexpression of Sae2 (Clerici et al., 2005b). MR(R20M)X when combined with Sae2 showed a

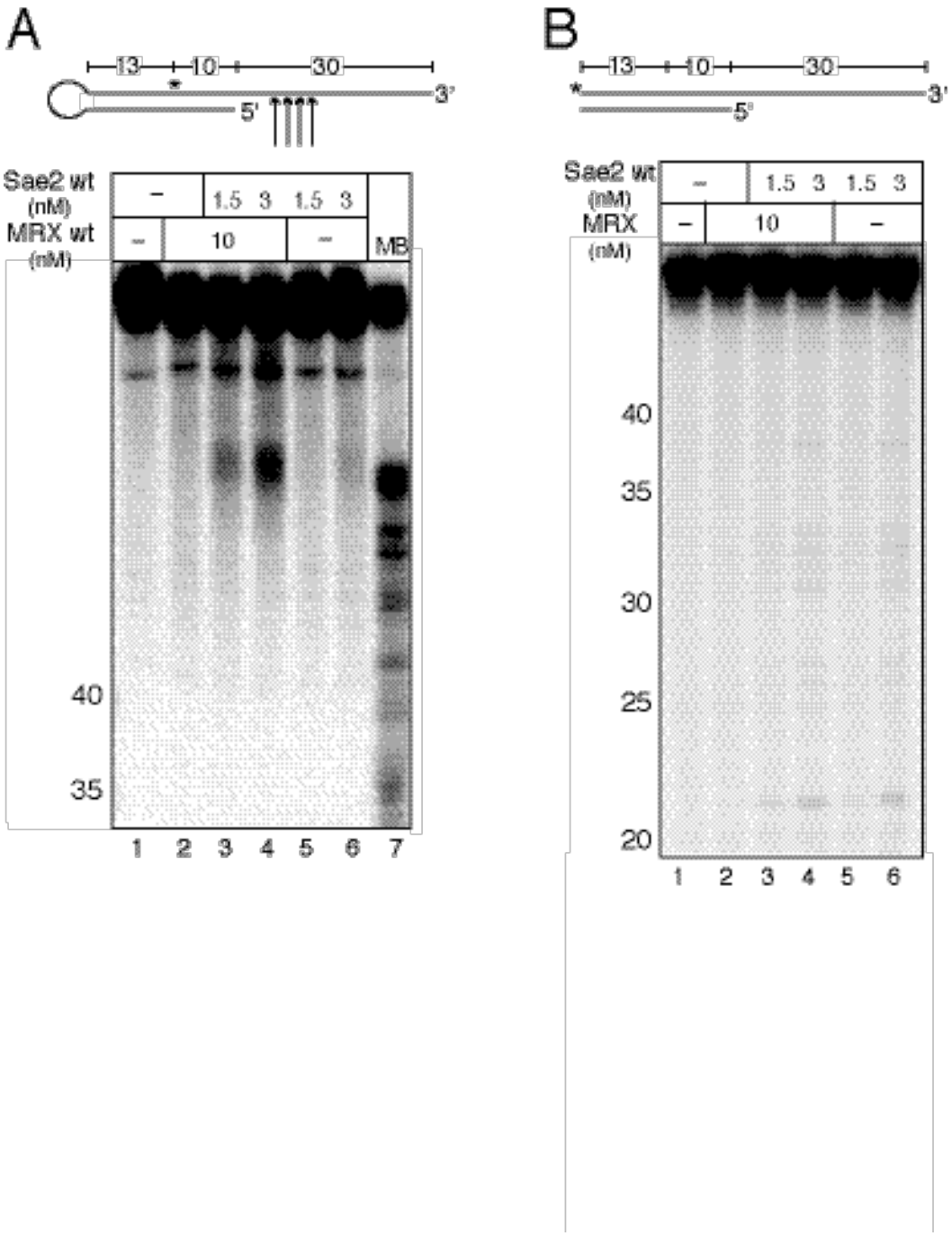

Figure 3.8 MRX and Sae2 cooperative endonuclease activity on hairpin structures in magnesium. A) The MRX complex stimulates Sae2 endonuclease activity on the ssDNA adjacent to a hairpin structure. MB; mung bean nuclease. B) Sae2 and MRX did not exhibit nuclease activity on a DNA substrate analogous to the hairpin substrate in (A) except lacking a hairpin structure.

2-fold decrease in hairpin cleavage compared to wildtype MRX and Sae2 (Figure 3.9B lanes 9-11). This suggests that MR(R20M)X can act cooperatively with Sae2 but exhibits a subtle defect in this functional interaction.

MRX EXONUCLEASE ACTIVITY CAN FACILITATE HAIRPIN REMOVAL BY SAE2

The hairpin cleavage assay above suggested that MRX stimulates Sae2 cutting of single-stranded DNA adjacent to a hairpin structure, independently of MRX nuclease activity. Yet Mre11 nuclease activity is clearly required in vivo for the processing of hairpin-capped DSBs (Lobachev et al., 2002). To resolve this contradiction, we considered the substrate specificity of Sae2 as shown in Figure 3.7A above, where we found that a ssDNA gap of 11-nucleotides allowed Sae2 cutting but a gap of 5-nucleotides did not. We hypothesized that one role of Mre11 exonuclease activity may be to widen gaps adjacent to hairpins to allow Sae2 endonuclease activity. To test this idea, an internally labeled hairpin was constructed containing an 8-nucleotide loop hairpin and fully paired hairpin at either end, with a 5-nucleotide gap between the 3' and 5' ends. When MRX was incubated with this substrate in manganese, the gap was widened due to MRX 3'→5' exonuclease digestion (Figure 3.10A). When Sae2 was added in a second step with magnesium to inhibit exonuclease activity by MRX, cleavage in the ssDNA region was observed which was dependent on

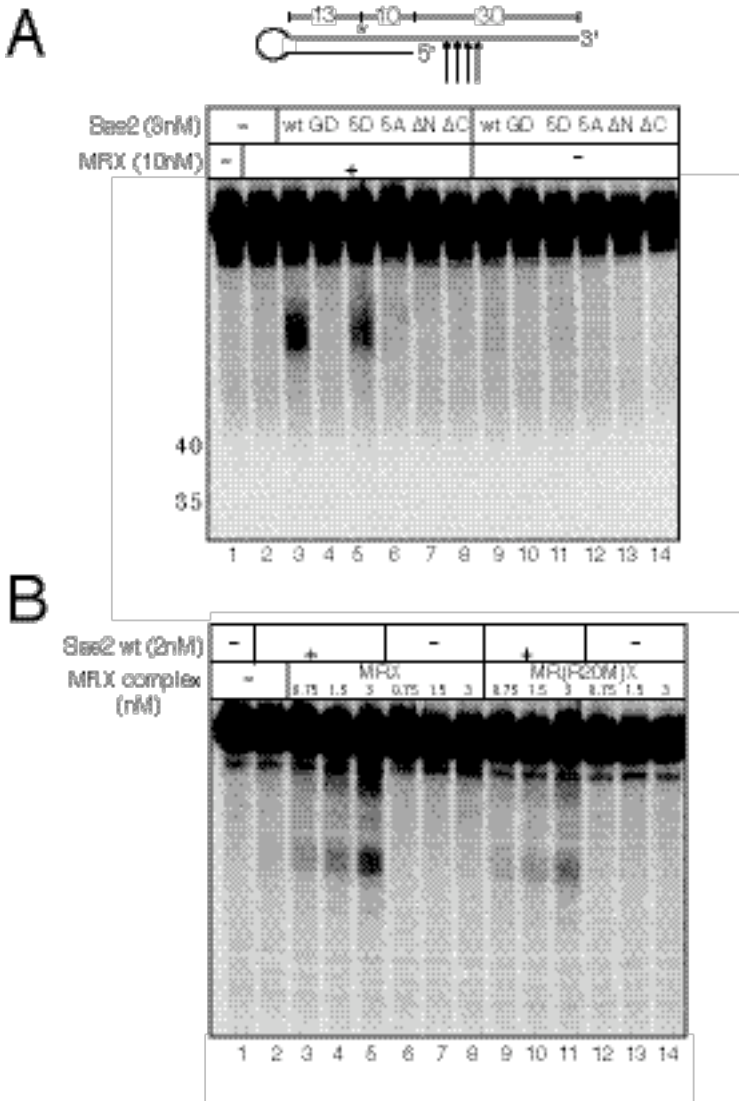

Figure 3.9 Characterization of mutant Sae2 and MRX complexes on the hairpin structure. A) The Sae2(5D) protein was stimulated by MRX to cleave the hairpin structure at the ssDNA overhang but Sae2(G270D), Sae2(5A), Sae2(ΔN) and Sae2(ΔC) proteins were not stimulated by MRX. B) MR(R20M)X mutant complex stimulated Sae2 endonuclease activity on the hairpin structure 2-fold less efficiently compared to than wildtype MRX.

both MRX and Sae2 (Figure 3.10A, lanes 2-7). Therefore, MRX stimulates Sae2 endonuclease cutting on substrates that contain gaps that are too small to facilitate Sae2 cutting in the single-stranded region. Since MR was also able to stimulate Sae2 in magnesium we tested the nuclease deficient M(D16A)R and Mre11-3/R complexes with this hairpin structure. Neither the M(D16A)R nor the Mre11-3/R complexes exhibited exonuclease activity (Figure 3.10B, lanes 5-10), thus Sae2 was unable to cut the substrate (Figure 3.10B, lanes 11-12).

SAE2 NUCLEASE ACTIVITY

As shown above with the hairpin DNA substrates, Sae2 itself exhibits endonuclease activity on single-stranded DNA. To characterize this activity further, we tested wildtype Sae2 with a branched DNA structure as shown in Figure 6A. We observed that wildtype Sae2 generated several endonucleolytic products from this 5'-labeled substrate, all within the 15nt 5' flap region (Figure 3.11A lane 2-3). In addition, Sae2 also removed the radiolabed nucleotide at the 5' end of the 5' flap, and cleaved the DNA within the duplex region at several points, although the cutting within the duplex region was much less efficient compared to the cutting within the ssDNA region.

The Sae2 mutants were also tested with this substrate which showed that they exhibited varying degrees of activity (Figure 3.11A, lanes 4-13). The

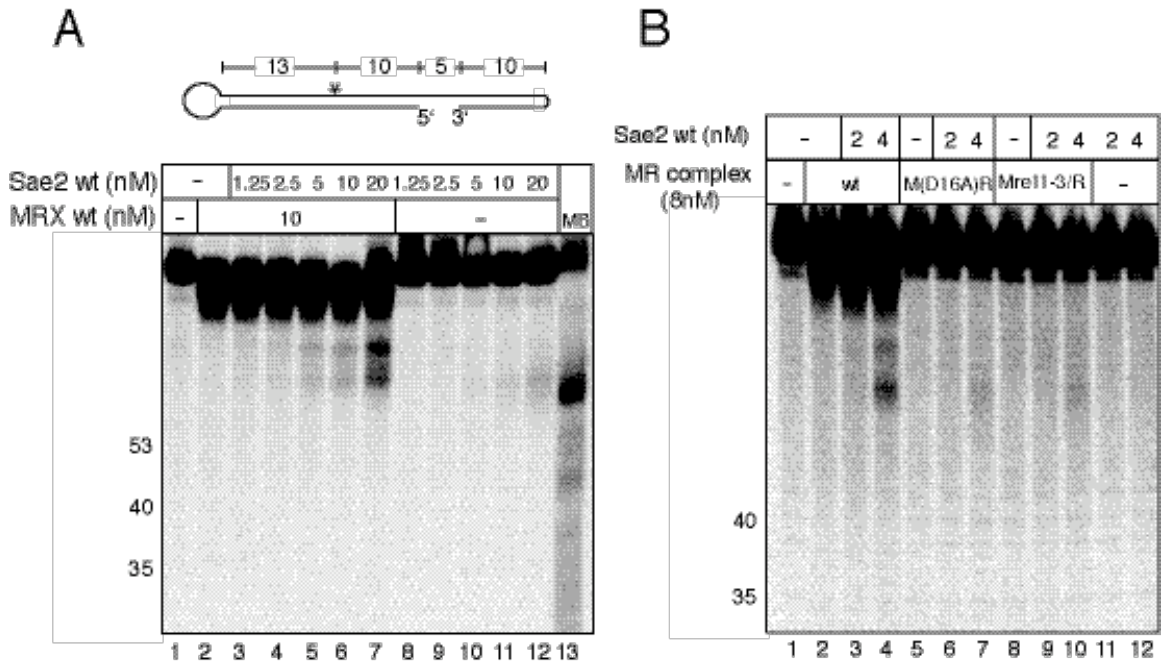

Figure 3.10 MRX exonuclease activity contributes to removal of hairpins. A) An internally labeled double-ended hairpin structure with a 5-nucleotide gap between 3' and 5' end was constructed. MRX was incubated with the hairpin substrate in manganese for 20 minutes. Magnesium and the Sae2 protein was added and incubated for 30 minutes. Exonuclease activity of MRX stimulated the Sae2 protein to cleave the hairpin at the widen once it was widened. B) M(D16A)R and Mre11-3/R did not exhibit exonuclease activity and therefore were unable to stimulate Sae2 endonuclease activity.

Sae2(G270D) and Sae2(ΔN) mutants exhibited essentially no endonuclease activity on this substrate (Figure 3.11A, lanes 4-5 and 10-11). The Sae2(5D) mutant showed similar activity as wildtype Sae2 (Figure 3.11A, lanes 6-7), while the Sae2(5A) and Sae2(ΔC) mutants both showed intermediate levels of endonuclease activity (Figure 3.11A, lanes 8-9 and 12-13). Interestingly, the Sae2(5A) and Sae2(ΔC) mutants did not show any nuclease activity with MRX on the hairpin substrate (Figure 3.9A) but were able to cleave the single-stranded DNA in the branched substrate. The Sae2(5D) mutant is the only mutant which shows similar levels of endonuclease activity on the branched substrate compared to the hairpin substrate.

To test the DNA structure requirements for Sae2 endonuclease activity, complementary strands were annealed to either the 5' or 3' flap of the branched substrate yielding a total of 5 substrates numbered as in Figure 3.11B. When the branched DNA included a complementary strand that fully paired with the 5' flap (Substrate 2), Sae2 no longer cut along the 5' flap or removed the 5' label but showed enhanced cutting within the duplex region (Figure 3.11B, lane 4). In contrast, Sae2 cut Substrate 3, which contained a complementary strand annealed to the 5' flap but also a 4-nucleotide gap at the single strand-double strand junction, with a pattern similar to the pattern seen with Substrate 1 (Figure 3.11B, lane 6). Substrates 4 and 5 are analogous to Substrates 2 and 3,

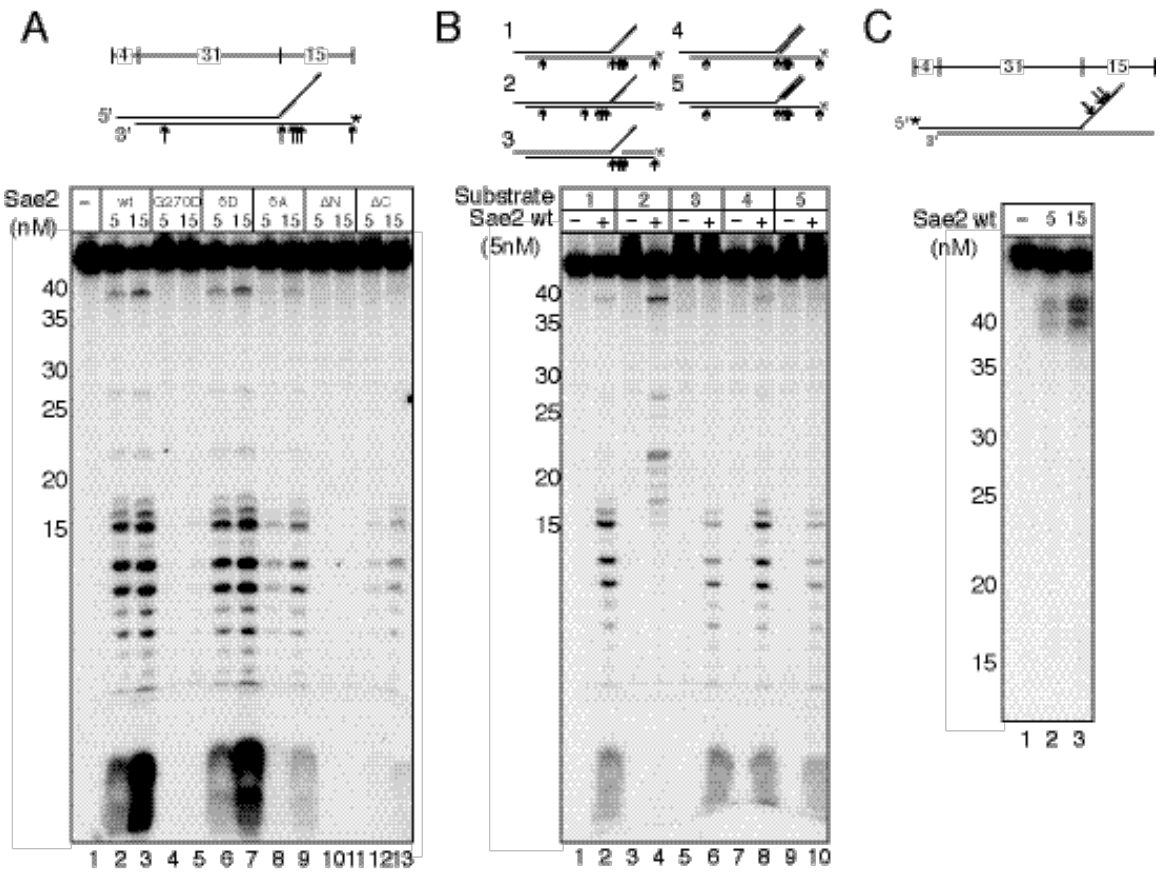

Figure 3.11 Characterization of the Sae2 protein endonuclease activity on branched DNA substrates. A) The wildtype Sae2 protein exhibited endonuclease activity on ssDNA and minor activity on the DNA duplex region of a branched substrate. Sae2(G270D) (lanes 4-5), Sae2(5D) (lanes 6-7), Sae2(5A) (lanes 8-9), sae2(ΔN) (lanes 10-11) and Sae2(ΔC) (lanes 12-13) mutant proteins were tested for endonuclease activity and compared to wildtype. B) Wildtype Sae2 exhibit different endonucleolytic cleavage patterns on the branched substrate with complementary strand annealed to the 5' or 3' flaps. Substrates 2 contained complementary DNA strands fully paired to the 5' flap of the branched substrate (lanes 3-4). Substrates 3 contained complementary strands to the 5' flap including a 4-nucleotide gap at the ss-dsDNA junction (lanes 5-6). Substrates 4 and 5 were analogous to substrates 2 and 3 except the complementary strands were annealed to the 3' flap (lanes 7-8 and 9-10). C) The Sae2 protein also exhibited endonuclease activity on the 3' flap of the branched substrate.

respectively, except the complementary strands were annealed to the 3' flap. The Sae2 endonuclease cleavage pattern was similar for substrates 1, 3, 4 and 5, in which all the substrates were cut at the junction and along the 5' flap (Figure 3.11B, lanes 2, 6, 8, and 10). Labeling of the top strand of the branched substrate, as shown in Figure 3.11C, showed that Sae2 also cut the top strand, exclusively within the 3' flap region (lanes 2-3), but not at the base of the 3' flap. Taken together, these data demonstrate that Sae2 is an endonuclease with a strong preference for single stranded DNA and single strand-double strand junctions but is also able to cut duplex DNA at a low level.

SENSITIVITY OF SAE2 MUTANT STRAINS TO MMS

To determine the in vivo effects of the Sae2 mutant proteins we have characterized, we expressed each mutant in *S. cerevisiae* from a low-copy *CEN* plasmid as a C-terminal Calmodulin Binding Protein (CBP) under the control of the endogenous *SAE2* promoter. We first tested the survival of *Δsae2* strains expressing the Sae2 mutant proteins after exposure to the radiomimetic alkyating agent methyl-methane-sulfonate (MMS) (Figure 3.12). Wildtype Sae2 was able to fully complement a *Δsae2* when exposed to MMS and *Δsae2* showed extreme sensitivity even at low concentrations of MMS. Also, to determine if the recombinant Sae2 is functional in vivo, we transformed a plasmid

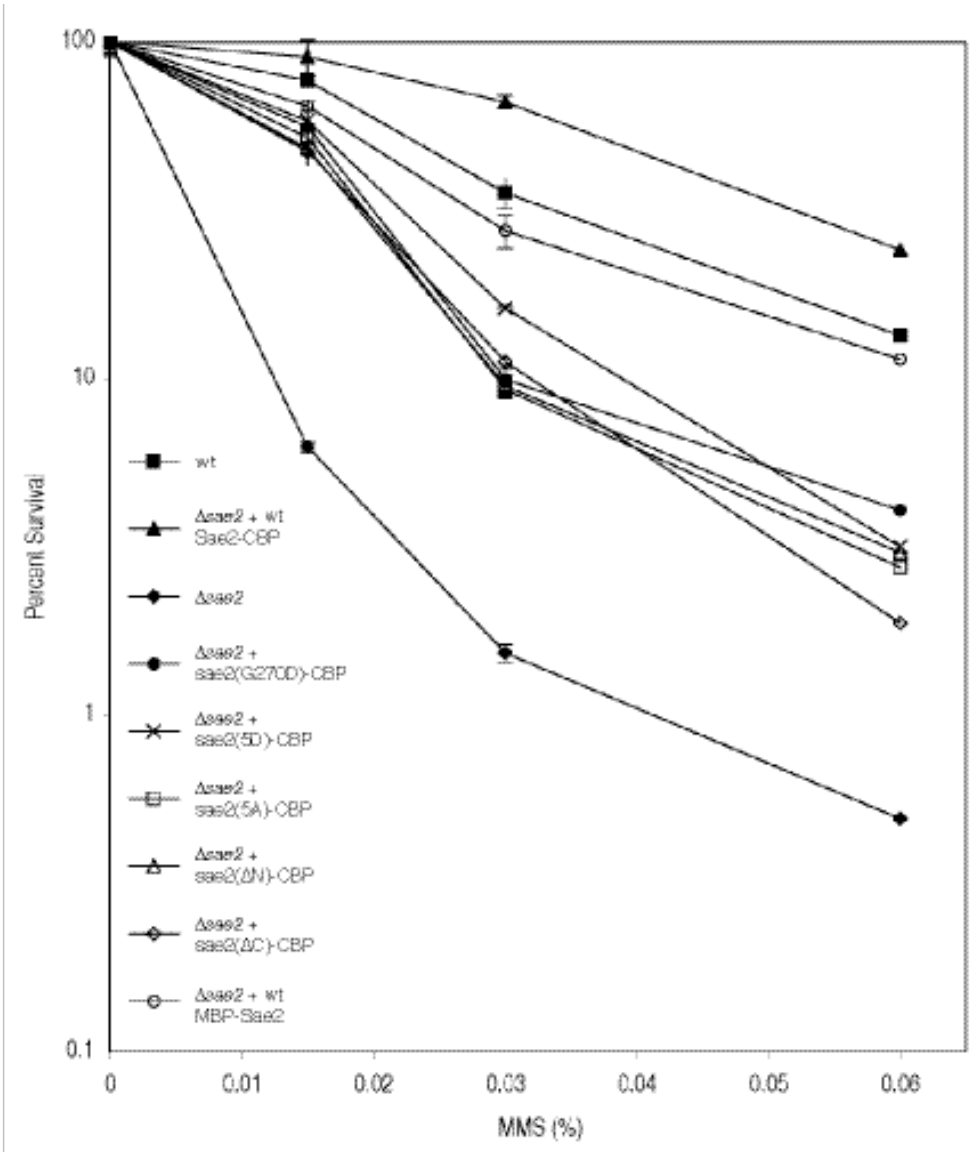

Figure 3.12 MMS survival of Sae2 mutants. Wildtype Sae2 (both CBP and MBP tagged), Sae2(G270D), Sae2(5D), Sae2(5A), Sae2(ΔN), and Sae2(ΔC) proteins were expressed from *CEN ARS URA3* containing plasmids in *Δsae2* strains. Survival of each strain was tested with MMS at 0.015, 0.03, and 0.06% in liquid media for 5 hours and then washed and plated.

containing MBP-*SAE2* under control of the *HIS3* promoter (gift A. Rattray) into the *Δsae2*. We established that MBP-Sae2 also completely complements for the loss of endogenous Sae2. Therefore the presence of a C-terminal CBP tag or a N-terminal MBP tag does not affect Sae2 function in vivo. Several mutants including *sae2(G270D), sae2(5D), sae2(5A), sae2(ΔN)*, and *sae2(ΔC)* were tested for complementation of the *Δsae2* strain after exposure to MMS. All mutant strains showed intermediate sensitivity to MMS when compared to wildtype and *Δsae2* strains.

THE EFFECT OF SAE2 MUTANTS ON RECOMBINATION BETWEEN INVERTED REPEATS

Lobechev et al. demonstrated that the MRX and Sae2 complexes are required for resolution of hairpin-capped DSBs in vivo (Lobachev et al., 2002). Large inverted *Alu* repeats inserted into the *LYS2* gene can extrude and form cruciform structures (Figure 1.4B). The cruciform is cleaved, in a MRX-independent manner, at the base leading to hairpin-capped DNA ends. The MRX and Sae2 complexes were required to remove the hairpin-capped ends to allow for homologous recombination repair. In *Δmrx* and *Δsae2* strains the hairpin-capped DSBs lead to gene amplification. To investigate the effect the Sae2 mutants have on DNA hairpin resolution we tested recombinational rate of each mutant strains containing the inverted *Alu* repeats. Wildtype Sae2 complemented *Δsae2* similar to previous reports for wildtype strains

Table 3.1

Strain	Recombination rate (per 10^7 cells)[a]
Sae2-CBP wt	3874 (±1379)
null (pRS316)	52 (±12)
sae2(G270D)-CBP	77 (±8)
sae2(5D)-CBP	93 (±62)
sae2(5A)-CBP	68 (±19)
sae2(ΔN)-CBP	69 (±30)
sae2(ΔC)-CBP	40 (±4)

[a] Recombination rate induced by *Alu* repeats in wildtype (TP2479), *null* (TP2478), *sae2(G270D)-CBP* (TP2484), *sae2(5D)-CBP* (TP2803), *sae2(5A)-CBP*(TP2482), *sae2 (ΔN)-CBP* (TP2848) and *sae2(ΔC)-CBP* (TP2482) were determined as described in Chap. 2. Standard deviations are given in parentheses.

(Baroni et al., 2004; Lobachev et al., 2002) (Table 3.1). *sae2(G270D), sae2(5D), sae2(5A), sae2(ΔN),* and *sae2(ΔC)* mutant strains all showed similar deficiency in hairpin-capped DSB resolution as *Δsae2* strains (Table 3.1).

CHAPTER 4: REPAIR OF TOPOISOMERASE II CONJUGATES

INTRODUCTION

DNA topoisomerases are essential enzymes required to control topology of DNA helices during cellular processes such as replication, transcription, recombination, and chromosome segregation. Topoisomerase enzymes are categorized into two groups: type I and type II (Wang, 2002). Type I topoisomerases are further divided into type IA and IB subgroups, while type II topoisomerases are also divided into type IIA and IIB. Each topoisomerase subgroup is structurally and mechanistically distinct but most topoisomerase enzymes serve to control superhelical strain within DNA molecules.

Yeast topoisomerase I (TOPOI) is an example of the type IB group. Type I topoisomerases bind duplex DNA and unpair a short region (Wang, 2002). Topoisomerase I breaks a single DNA backbone by creating a phosphodiester bond between the enzyme and the 3' end of the DNA molecule. The DNA duplex is relaxed and the topoisomerase enzyme rejoins the nicked ssDNA ends and is released.

To relax DNA, most type II topoisomerases also form phosphodiester bonds between the DNA backbone and the enzyme. However, the covalent linkage is between the 5' end of the DNA molecule and the topoisomerase II protein (Wang, 2002). Both DNA strands in a duplex are broken by a dimer forming 5'

DNA-protein linkages to allow another DNA duplex to pass through the broken DNA duplex.

Most of the type II topoisomerase enzymes transiently break dsDNA and rejoin the DNA ends, with the exception of Spo11. Spo11 is a putative member of the type IIB subfamily of enzymes and shares homology to the A subunit of topoisomerase VI from *S. shibatae* (Wang, 2002). In vivo data shows that Spo11 catalyzes meiotic DSB formation and forms covalent linkages at the 5' ends of the DNA molecule (Keeney et al., 1997). The MRX and Sae2 complexes are required for the removal of Spo11 and repair of meiotic DSBs (Alani et al., 1990; Neale et al., 2005).

Several cancer therapeutic drugs, known as topoisomerase poisons, can bind the topoisomerase molecules and prevent religation of the DNA ends, thus creating covalent protein-DNA lesions ("cleavable complexes")(Wang, 2002). DNA-topoisomerase complexes are then transformed into potentially lethal single-stranded or double-stranded DNA breaks when DNA replication or transcription machinery collide with the protein-DNA complexes. The topoisomerase poisons can be utilized to determine the necessary cellular components to repair topoisomerase-mediated DNA damage.

Topoisomerase I-DNA conjugates are potentially lethal DNA lesions and must be removed before DNA repair can be completed. Tyrosyl-DNA phosphodiesterase (Tdp1p) specifically removes topoisomerase I by breaking the

bond between the tyrosine in the enzyme and the 3' phosphate in the DNA backbone (Pouliot et al., 1999). The substrate specificity of Tdp1p suggests that Tdp1p only contributes to removal of topoisomerase I-DNA conjugates after the lesions have been converted to DSBs (Pouliot et al., 1999).

The endonuclease Rad1/Rad10 is involved in nucleotide excision repair and has been identified in an alternative pathway to repair topoisomerase I DNA lesions (Liu et al., 2002; Vance and Wilson, 2002). Rad1/Rad10 is a structure-specific endonuclease that cleaves branched DNA structures at DSBs. Therefore unlike Tdp1, Rad1/Rad10 cleaves a branched substrate to remove the topoisomerase I conjugate. Also, MRX and Sae2 nuclease functions were found to be in another parallel pathway to remove topoisomerase I conjugates (Deng et al., 2005); although the mechanism by which MRX and Sae2 function in topoisomerase I conjugate removal is not understood.

Initially it was reported that TdpI was not involved in removal of topoisomerase II conjugates on DNA 5' ends (Nitiss et al., 2006). However, Nitiss and colleagues recently reported that recombinant yeast Tdp1 protein exhibited enzymatic activity on a 5'-linked oligopeptide substrate, which simulated a topoisomerase II linked to DNA, by breaking the peptide-DNA bond and removing the peptide (Nitiss et al., 2006). Deletion of *TDP1* also confers sensitivity to topoisomerase II poisons, such as etoposide. This suggests that Tdp1 is at least partially responsible for the repair of topoisomerase II DSBs.

Repair of topoisomerase II-meditated DNA damage involves the homologous recombination pathway. Deletion of either *RAD50* or *RAD52* genes leads to hypersensitivity to etoposide (Nitiss et al., 2006; Sabourin et al., 2003). Although the Tdp1 protein can cleave the tyrosine-phosphate bond between topoisomerase II and DNA, Keeney and colleagues demonstrated that the DNA upstream of the topoisomerase II conjugate is cleaved, leaving a short ssDNA molecule attached to the enzyme (Neale et al., 2005). This suggests that the primary repair pathway responsible for the removal topoisomerase II conjugates cleaves the DNA upstream of the topoisomerase II conjugate and therefore leaves clean DNA ends. The exact nucleases involved in removing the topoisomerase II conjugates are still unknown. Interestingly, Spo11 conjugates formed during meiosis were also shown in this study to be removed with a short DNA molecule attached. The removal of Spo11 was MRX and Sae2 dependent (Neale et al., 2005).

Studies preformed by Nitiss and colleagues (John Nitiss, personal communication) tested the sensitivity of *Δmre11* and nuclease-deficient *mre11* strains when exposed to topoisomerase II poisons. When compared to wildtype and *Δmre11* strains, the nuclease deficient *mre11-H125N* strain exhibited intermediate sensitivity to TOP-53, a derivative of etoposide. In addition, overexpression of topoisomerase II in *Δsae2* strains conferred an increase in sensitivity to etoposide when compared to wildtype strains. These studies

suggest that the MRX and Sae2 complexes are involved in the repair of topoisomerase II conjugates, although their role in this process is poorly understood and may be partially redundant with Tdp1 and perhaps other cellular enzymes. In contrast, the removal of Spo11 conjugates is clearly MRX and Sae2-dependent.

RESULTS

The phenotype of the *mre11* nuclease-deficient, *Δmre11*, and *Δsae2* strains when exposed to topoisomerase poisons suggests that the MRX and Sae2 complexes are involved in topoisomerase II conjugate removal. Prior to purifying recombinant MRX and Sae2 complexes we attempted to address the possibility that the Mre11 complex is required for the removal of topoisomerase II conjugates by utilizing human MRN complex. Covalent topoisomerase II-DNA complexes were prepared utilizing recombinant human topoisomerase IIα (Worland and Wang, 1989) and oligonucleotide or plasmid DNA molecules. To generate the conjugates, topoisomerase IIα was incubated with the DNA substrate in the presence of etoposide (Figure 4.1A, lane4 and Figure 4.2B, lanes 2 and 6). Supercoiled and open-circular plasmid species are well separated in a 1% agarose gel (Figure 4.1A). Removal of the topoisomerase IIα conjugate would appear as a linear band (Figure 4.1A, lanes 3 and 5 and Figure, 4.1B lanes 3 and 6). Human MRN protein was tested with the plasmid-

topoisomerase IIα substrate; however, our initial results show no direct processing of the conjugates with the MRN complex alone (Figure 4.1B, lane 5).

To examine the topoisomerase IIα conjugate with greater resolution, an internally labeled oligonucleotide substrate was designed so that when topoisomerase IIα was covalently linked to the 5' end of the cleavage site, the radiolabel remained attached to the conjugate (Figure 4.2A). To resolve the products, we used high percentage denaturing polyacrylamide gels. DNA released from the topoisomerase IIα conjugate and substrate DNA was resolved, although unprocessed conjugates did not enter the gel. Proteinase K was added to remove topoisomerase IIα (Figure 4.2B, lane 3). Neither human Mre11 nor MRN protein removed topoisomerase IIα from the DNA substrate (Figure 4.2B, lanes 4-5). Low percentage SDS-PAGE gels were also used to resolve the products after processing. The cleavable complex was able to enter the gel and processing of the conjugate would result in the disappearance of the complex (Figure 4.2C, compare lanes 2 to 3). The human MRN complex was also tested on the oligonucleotide-topoisomerase IIα substrate and again no direct processing has been seen (data not shown).

Wildtype human topoisomerase IIα formed a cleavable complex with the oligonucleotide substrate in the presence of etopoisode; however, the complex

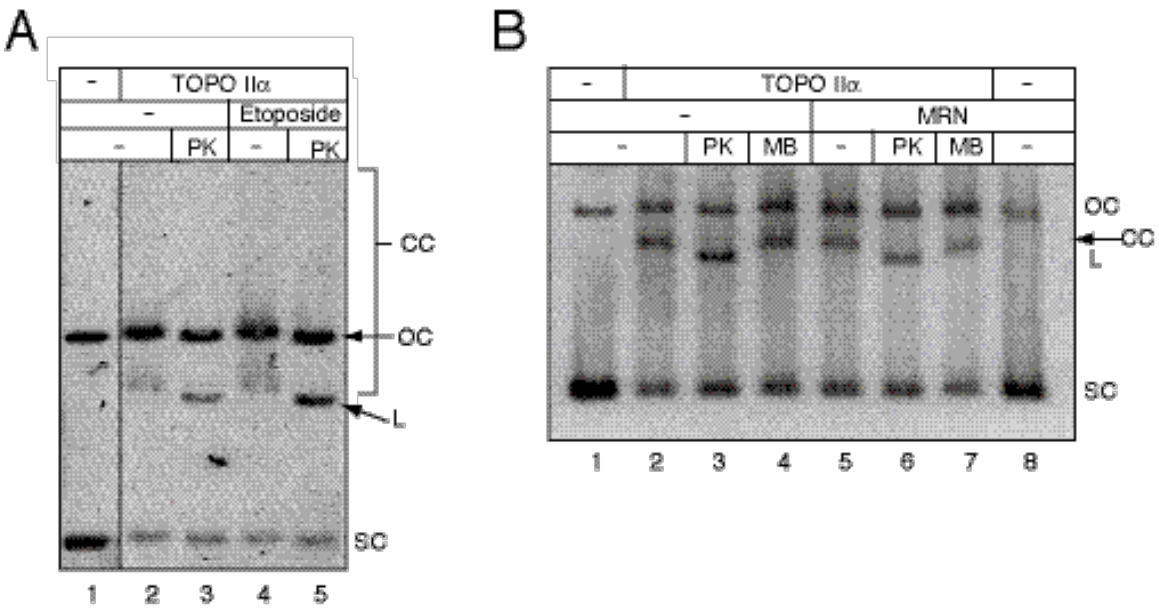

Figure 4.1 Topoisomerase II-plasmid DNA conjugates. A) TOPOII cleavable complexes were prepared with wildtype human topoisomerase IIα, supercoiled plasmid DNA with and without etoposide. Mobility shift of the DNA is visible with TOPOIIα. Linear DNA is visible when TOPOIIα was removed by proteinase K. B) Cleavable complexes were prepared as in (A). human MRN did not remove TOPOIIα (lane 5). Mung bean, a ssDNA endonuclease, did not remove TOPOIIα. PK, proteinase K; MB, mung bean; OC, open circular; CC, cleavable complex; L, linear; SC, supercoiled plasmid DNA.

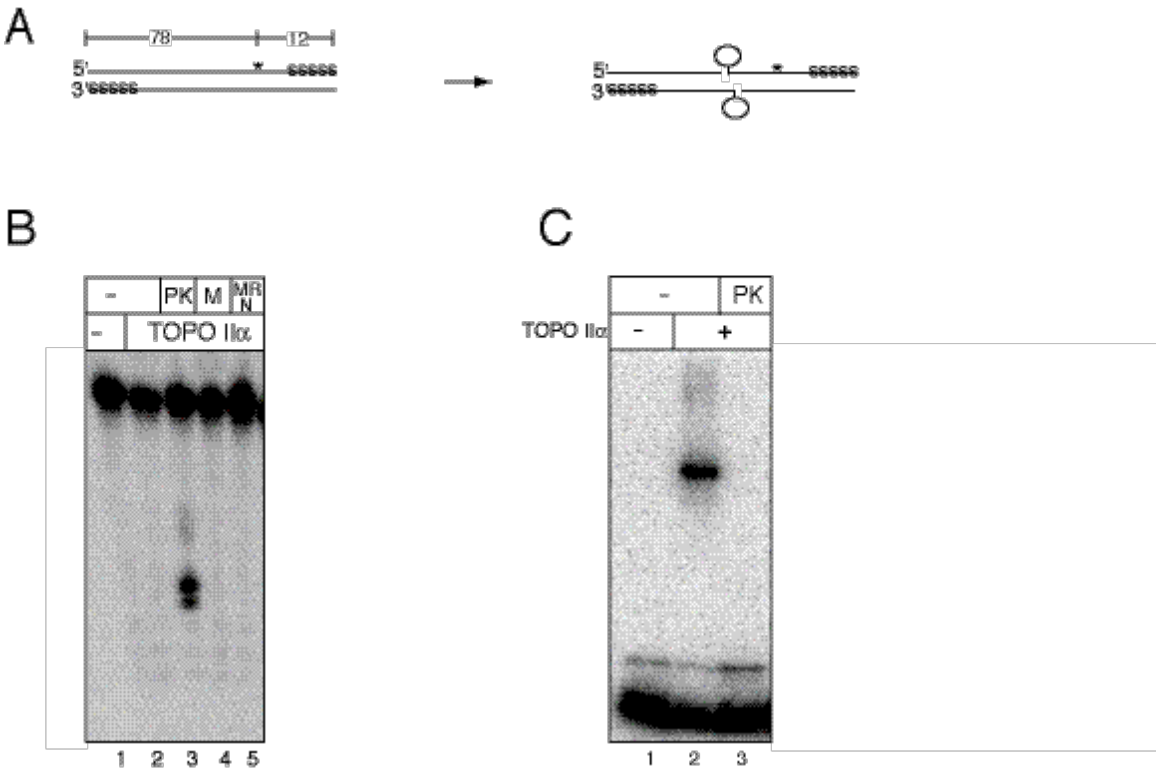

Figure 4.2 Topoisomerase II-oligonucleotide DNA conjugates. A) Internally labeled 90-nucleotide duplex DNA substrate was prepared. Addition of TOPOIIα is expected to form covalent linkages with 5' DNA ends at the cleavage sites. B) TOPOIIα cleavable complexes were tested with human Mre11 (M) and MRN complexes. Proteinase K (PK) removed TOPOIIα and released the labeled DNA fragment. Products were resolved in a 20% denaturing polyacrylamide gel. C) TOPOIIα cleavable complex forms higher mobility products in a SDS-PAGE gel. sssss, phosphorothioate bonds.

was disrupted with the addition of manganese, which is necessary for Mre11 nuclease activity. We later learned that topoisomerase II conjugates can be stabilized by a point mutation, S763W, in the presence of etoposide such that they are not disrupted by the addition of metal ions (data not shown). Preliminary tests of the purified mutant topoisomerase IIα protein demonstrated strong activity in $MnCl_2$. In future experiments mutant topoisomerase IIα will be used because it forms highly stable protein-DNA conjugates in the presence of manganese.

If the MRN(X) complex is involved in the removal of topoisomerase conjugates, it could be that it does not directly remove the conjugate from the DNA. An alternative possibility is that the MRN complex processes the DNA strand adjacent to the topoisomerase IIα adduct, which is the free 3' end. To investigate this possibility, the 5' end of an oligonucleotide substrate was labeled. On a 20% denaturing acrylamide gel we can visualize products formed as a result of the topoisomerase IIα cleavage and found that the 3' strand adjacent to the conjugate was degraded in the presence of the MRN complex (Figure 4.3, lanes 5 and 6). This shows that the MRN complex can access the free 3' ends adjacent to the topoisomerase IIα conjugate, and suggests that this may be part of the repair process.

This hypothesis will also be tested using the plasmid substrate. Mung bean nuclease, a single-stranded endonuclease, was incubated with the

conjugates after incubation with the MRN complex (Figure 4.1B, lanes 3 and 6). If the MRN complex was degrading from the 3' end and leaving single-stranded regions flanking the topoisomerase II conjugate then mung bean nuclease should be able to cleave the topoisomerase off the substrate. Experiments are still underway to understand the role of MRN(X) in processing topoisomerase-DNA complexes.

Alternatively, it is possible that the MRN complex does process topoisomerase IIα conjugates directly, but requires additional cofactor(s). In *S. cerevisiae*, Sae2 is a potential candidate for an Mre11 cofactor, considering the large body of evidence showing cooperative activities of Sae2 and the MRX complex on hairpin DNA structures (Lobachev et al., 2002; Rattray et al., 2001). *mre11* nuclease-deficient strains and *Δsae2* strains also have similar sensitivity to DNA damaging such as IR, MMS and bleomycin. Moreover, both *Δsae2* and *mre11* nuclease-deficient strains are deficient in Spo11 removal in meiosis (Keeney et al., 1997; Neale et al., 2005). Now that we have purified both MRX and Sae2 complexes we will be able to test their activity on topoisomerase IIα substrates to test this hypothesis.

Unpublished data from John Nitiss suggests Sae2 plays a role in vivo to repair topoisomerase II-mediated DSBs. To analyze this in greater detail we tested the survival of wildtype, *Δsae2*, *Δrad52*, and *Δsae2Δrad52* strains when

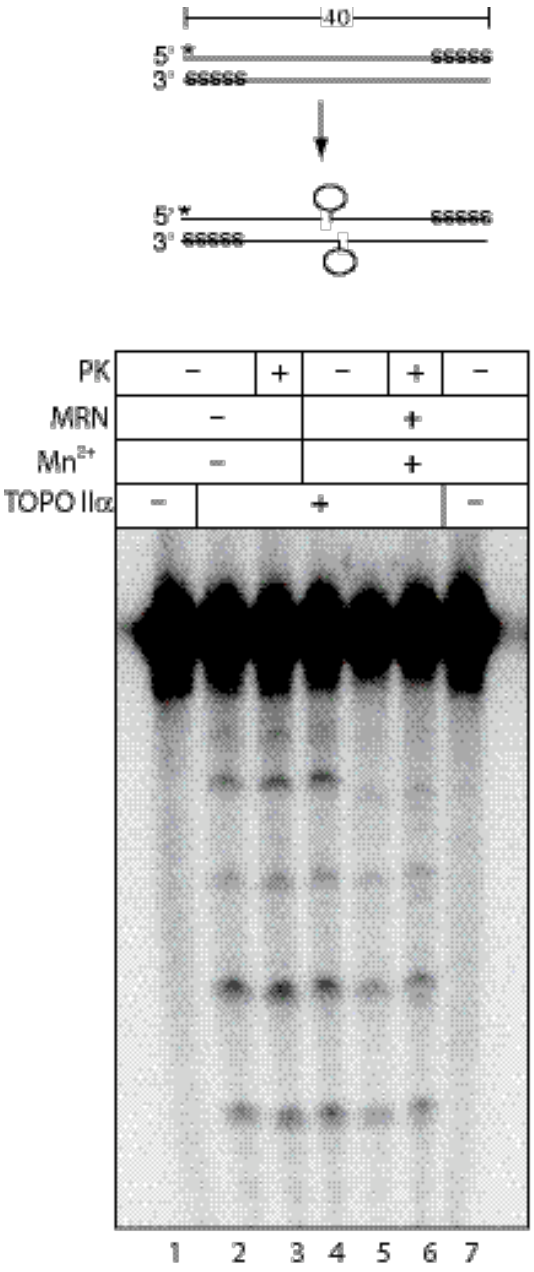

Figure 4.3 3'→5' exonuclease activity of MRN and the TOPOII-conjugate. A 40-nucleotide duplex DNA substrate labeled at the 5' end was used to prepare cleavable complexes. MRN was able to degrade the 3' end at the TOPOIIα cleavage site. sssss, phosphorothioate bonds.

exposed to amsacrine (m-AMSA) (Figure 4.4). m-AMSA is a topoisomerase II inhibitor that intercalates into duplex DNA and prevents completion of the topoisomerase II cycle. *Δrad52* strains were previously reported to exhibit extreme sensitivity to topoisomerase II poisons. In a double knockout, *Δrad52Δsae2*, all homologous recombination events will be eliminated by the absence of *RAD52* but Mre11 and Sae2 nuclease activities will remain intact. The non-homologous end joining pathway can be utilized only after topoisomerase II conjugates are removed. Preliminary data shows that *Δrad52Δsae2* exhibits greater sensitivity than *Δrad52* single mutant when exposed to m-AMSA, again suggesting that Sae2 may play a role in the removal of topoisomerase II conjugates. Sae2 mutant protein will also be tested in this assay to determine the domains necessary for topoisomerase II-induced DSB repair.

CONCLUSIONS

Topoisomerase II is essential for DNA replication, transcription and many other cellular functions to control DNA topology. In vivo data suggests that the MRX and Sae2 complexes are involved in removal of topoisomerase II conjugates. We have designed in vitro assays to investigate the role of MRX and

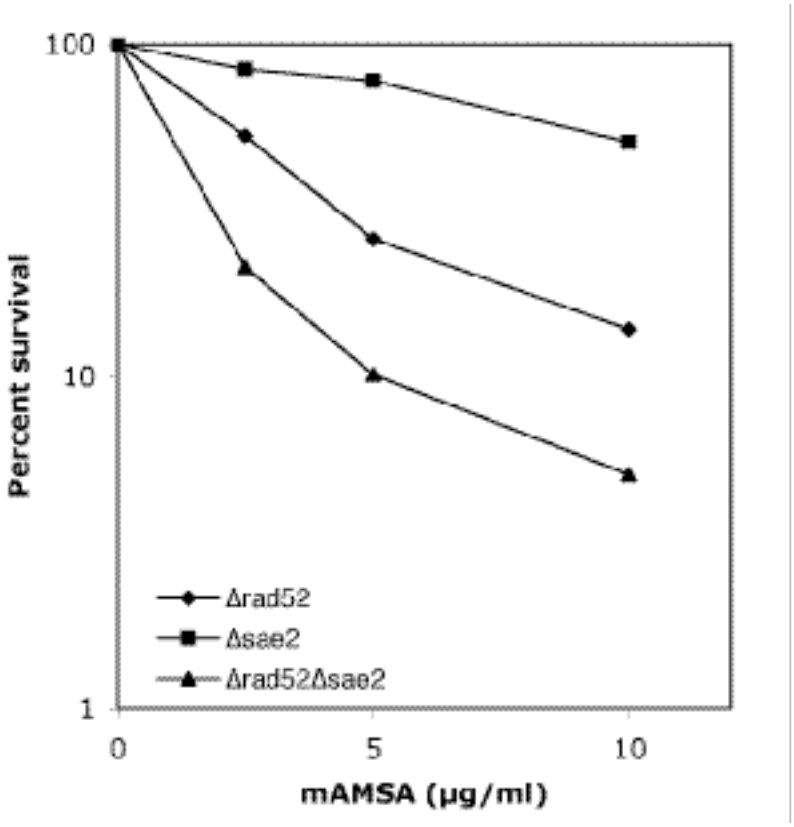

Figure 4.4 Δsae2 strains are sensitive to mAMSA, a topoisomerase II inhibitor. Δrad52, Δsae2 and Δrad52Δsae2 strains were tested for survival when exposed to m-AMSA.

Sae2 in the removal of topoisomerase II conjugates and repair of topoisomerase II-mediated DSBs.

Topoisomerase IIα cleavable complexes were prepared using supercoiled plasmid DNA and duplex oligonucleotide substrates. Presently, the human MRN complex has not shown any nuclease activity on topoisomerase IIα adducts. It could be possible that other cofactors yet to be identified may be required for the MRN complex to remove the conjugates. Since the MRX and Sae2 nuclease activities have been implicated in removal of topoisomerase IIα conjugates, we will test both complexes with the cleavable-complex substrates.

Keeney and colleagues demonstrated that topoisomerase II conjugates are removed with a short DNA molecule attached (Neale et al., 2005). This data shows that topoisomerase II is not removed by cleavage of the tyrol-phosphate bond as Tdp1p cleaves topoisomerase I from DNA. Considering that the MRN complex exhibits 3'→5' exonuclease activity on the free 3' end adjacent to topoisomerase IIα conjugates, removal of the conjugates may resemble removal of the hairpin as described above (Figure 3.10). Possibly the MRX complex will degrade the 3' DNA end adjacent to the topoisomerase II conjugate and expose ssDNA and stimulate the Sae2 protein to cleave the ssDNA. This cleavage would result in small DNA molecules attached to topoisomerase II. To test this hypothesis, MRX will be incubated with the cleavable complex in $MnCl_2$ to stimulate MRX exonulcease activity and the Sae2 protein will be added. If we

observe activity of MRX and Sae2 on these conjugates, the Sae2 mutant proteins described in Chapter 3 will also be utilized to characterize the activities of Sae2 on this substrate.

CHAPTER 5: CONCLUSIONS

MRX AND SAE2 IN DSB REPAIR

In *S. cerevisiae* the Mre11 complex is required for homologous recombination (Krogh and Symington, 2004). The role of the MRX complex in homologous recombination is not understood, but it has been shown to be specifically required for processing of DNA ends at double strand breaks during meiosis (Alani et al., 1990). In several nonnull *rad50* mutant strains, termed *rad50S*, it was discovered that the putative type II topoisomerase Spo11 was covalently linked to the 5' end of DSBs during meiosis I (Alani et al., 1990; Keeney et al., 1997). In constrast, *Δrad50* strains do not make DSBs during meiosis and Spo11 was not found on the DNA (Alani et al., 1990; Keeney et al., 1997). Therefore, the MRX complex is required for the initiation of Spo11-induced recombination during meiosis and also for the processing of the DSBs. Interestingly, deletion of *SAE2* resulted in Spo11 conjugates accumulating similarly as the *rad50S* strains during meiosis (McKee and Kleckner, 1997; Prinz et al., 1997). Thus the MRX and Sae2 complexes are both required in vivo for the repair of meiotic DSBs.

In both meiotic and mitotic cells, 5' ends of DSBs are resected prior to homologous recombination (Krogh and Symington, 2004). However, the Mre11 complex from several organisms has been shown to exhibit 3'→5' exonuclease

activity in vitro, the opposite polarity of what is needed to make 3' ssDNA tails (Connelly et al., 1999; Paull and Gellert, 1998; Trujillo and Sung, 2001). It has been suggested that Sae2 may alter the nuclease activity of the MRX complex to process damaged DNA ends and achieve 5' resection.

Recombination fildelity is essential to ensure error-free repair during homologous recombination. Rattray et al., demonstrated that the Sae2 and MRX complexes were involved in accurate intrachromosomal homologous recombination at a site-directed DSB between two inverted repeats (Rattray et al., 2001). Foldback of a DNA end, due to small (4-9 nucleotides) inverted repeats, at the site-directed DSB leads to chromosomal instability and to gene amplification (Figure 1.3) (Rattray et al., 2005). The MRX and Sae2 complexes prevent gene amplification, possibly by processing the foldback structure. Lobechev et al. also demonstrated that large inverted repeats can form cruciform structures which also lead to chromosome instability (Lobachev et al., 2002). The cruciform structures are cleaved at the base and result in hairpin-capped DSBs (Lobachev et al., 2002). In the absence of the MRX and Sae2 complexes, these hairpin-capped DSBs accumulate and lead to large-scale chromosomal amplifications (Figure 1.4)(Lobachev et al., 2002). To investigate the role of the Sae2 protein in DSB repair we purified recombinant Sae2 protein as well as recombinant MRX and characterized their activities in vitro.

RECOMBINANT SAE2 PROTEIN

The Sae2 protein has no known homologs outside of fungi and no obvious motifs to suggest the function(s) of the Sae2 protein. Recombinant Sae2 protein with 6xhistidine and MBP on the N-terminal end was expressed and purified from *E. coli*. The final gel filtration step in the purification procedure yielded three distinct multimeric forms of recombinant Sae2 (Figure 3.1A). Preliminary sedimentation results suggest the protein in the first peak forms large aggregates while the second peak forms dimer and tetramer complexes (date not shown).

SAE2 PROTEIN EXHIBITS DNA BINDING ACTIVITY

The MRX and Sae2 complexes localize at the sites of DNA damage independently of each other (Lisby et al., 2004). We show here that the Sae2 protein exhibits DNA binding activity, suggesting that Sae2 foci are formed by a direct interaction between that protein and DSBs (Figure 3.4A). To investigate the effects of the MRX complex on the DNA binding activity of the Sae2 protein, we incubated the MRX and Sae2 complexes with the dsDNA substrate. Both complexes exhibited independent DNA binding activity, however, together the MRX and Sae2 complexes form higher order complexes than either protein alone (Figure 3.4B). One Sae2 mutant protein was unable to form higher order complexes with MRX and DNA even though it was able to form complexes

independently, suggesting the MRX and Sae2 complexes are interacting on the DNA substrate.

SAE2 STIMULATES MRX EXONUCLEASE ACTIVITY

In vivo data suggests that the MRX and Sae2 complexes function together processing DNA ends. To investigate if the Sae2 protein alters Mre11 nuclease activity we tested exo- and endonuclease activities of MRX in the presence and absence of Sae2. The MRX complex has previously been shown to exhibit mangaese-dependent 3'→5' exonuclease activity (Trujillo et al., 2003; Trujillo and Sung, 2001). Here we demonstrate that the Sae2 protein stimulates MRX nuclease activity by 2-4-fold on a 3' recessed DNA substrate (Figure 3.5A). The Sae2 protein by itself did not exhibit any nuclease activity on this substrate.

MRX AND SAE2 PROTEINS COOPERATIVELY REMOVE HAIRPIN STRUCTURES

As previously reported, both the MR and MRX complexes exhibited endonuclease activity on DNA hairpins, cutting at the tip or within the hairpin loop (Trujillo et al., 2003; Trujillo and Sung, 2001). We observed this with MR and MRX as well, and found that the concentration at which the MR complex exhibited hairpin cleavage is at least 10-fold higher than what is required for exonuclease activity (Figure 3.6, compare lanes 2 and 5). Addition of the Sae2 protein under these conditions in manganese did not stimulate hairpin cutting by the MR complex. At lower concentrations of the MR complex, there was no

detectable cleavage of the hairpin at the tip. Interestingly, addition of the Sae2 protein resulted in cleavage of the 3' overhang of the DNA hairpin substrate (Figure 3.6, lanes 8-9). Cooperative stimulation of removal of the 3' overhang was observed when both complexes were present (Fig. 3.7A, lanes 2, 3, and 9).

These results showed that the Sae2 protein exhibits nuclease activity. To investigate the substrate requirements for the Sae2 nuclease activity we constructed several DNA hairpin substrates with various 3' overhang structures. The Sae2 protein cleaved the ssDNA overhang adjacent to the hairpin structure and cleavage was inhibited when a complementary strand was present (Figure 3.7A). The length of single-stranded DNA adjacent to the hairpin proved to be important for Sae2 cleavage. When an 11-nucleotide gap was present adjacent to that hairpin stem Sae2 cut the substrate although much less than on the substrate lacking a complementary strand. When only a 5-nucleotide gap was present, cleavage by the Sae2 protein was nearly abolished. In comparison, the Sae2 protein showed 10-fold lower endonuclease activity on identical substrates lacking hairpin loops (Figure 3.7A and B). This suggests that removal of DNA hairpins from ends requires Sae2 endonucleolytic activity on single-stranded DNA regions adjacent to the hairpin structures and that the hairpin structures are required for this activity.

In their study analyzing the genetic requirements for removal of hairpin-capped DNA ends, Lobachev et al. embedded inverted *Alu* repeats into the *LYS2*

gene and truncated a second copy of the *lys2* gene on a separate chromosome (Lobachev et al., 2002). Inverted repeats have the potential to form cruciforms, which are known to generate DSBs at a 1000-fold higher rate compared to non-cruciform DNA (Lobachev et al., 2002). Lobechev et al. found that the cruciforms are cleaved at the base in a MRX and Sae2-independent manner. However, in *Δmrx*, *rad50S*, *mre11* nuclease-deficient, or *Δsae2* strains, a hairpin-capped DSB was found stabilized at the location of the cruciform. Based on this data one hypothesis is that, instead of cleaving the hairpin at the tip, the MRX and Sae2 complexes remove the hairpin structure by cleaving at the nick or gap adjacent to the hairpin structure.

We showed that the Sae2 protein cleaves the ssDNA adjacent to a hairpin structure (Figure 3A), although at 3-12-fold lower concentrations, the Sae2 protein showed cooperative activity with the MR complex (Figure 3.8A). The MRX and Sae2 complexes cleaved single-stranded DNA adjacent to the DNA hairpin structure cooperatively at 16-fold lower concentrations of the MRX complex than is required for endonucleolytic cleavage at the hairpin tip (Figure 3.6 compared to Figure 3.8A). This activity was observed in either manganese or magnesium, suggesting that the Sae2 protein is responsible for removing the hairpin and not Mre11 nuclease activity since Mre11 nuclease activity is manganese-dependent. Although the cleavage product is similar to the mung bean product, which is cut at the tip of the hairpin, results from Figure 3.7A

suggest the single-stranded DNA adjacent to the hairpin structure is cleaved. To verify the requirement for the DNA hairpin structure adjacent to ssDNA for MRX and Sae2 cooperative activity, we show that only minor cutting occurs on an identical substrate lacking the hairpin loop. Considering both Rattray et al and Lobechev et al in vivo results, small and large hairpin structures are resolved by MRX and Sae2 (Lobachev et al., 2002; Rattray et al., 2001). To define the structural requirements for these reactions we plan to investigate the distance limits of the ssDNA from the hairpin structure. Also, to investigate if the Sae2 protein preferentially binds to DNA hairpin structures, we plan to perform DNA-binding competition assays.

In vivo, *rad50S* mutant strains are deficient in resolution of hairpin-capped DSBs and exhibit intermediate sensitivity to MMS compared to wildtype and *Δrad50* strains (Alani et al., 1990; Lobachev et al., 2002). Although the MR(R20M)X complex does stimulate Sae2 endonuclease activity, the activity is diminished only about 2-fold (Figure 3.9B). This suggests that the MR(R20M)X complex is competent to stimulate the Sae2 protein, at least under the conditions of our in vitro assay. The nuclease assays are performed at 50mM NaCl. At more physiological conditions (100 or 150mM salt) interaction between Sae2 and the MR(R20M)X complex can possibly be diminished and reflect in vivo results where overexpression of Sae2 can partially complement deficiencies in SSA in *rad50S* strains (Clerici et al., 2005b).

Sae2 endonucleolytic activity was completely blocked when the ssDNA adjacent to the hairpin was only 5 nucleotides in length but not when the gap was 11 nucleotides (Figure 3.7A). Therefore, we hypothesize that at a nick or gap adjacent to a hairpin-capped DNA end, Mre11 exonuclease activity can widen the gap facilitating the Sae2 endonuclease activity to cleave the ssDNA . To investigate the role of Mre11 exonuclease activity in removal of hairpin-capped ends we constructed a double-ended hairpin DNA substrate with a 5-nucleotide gap between the 3' and 5' ends as shown in Figure 3.10A. The MRX complex was incubated first with the double-ended hairpin substrate in manganese to allow for 3'→5' exonuclease activity that widens the gap between the 3' and 5' ends of the substrate. Second, the Sae2 protein was added along with magnesium to inhibit any further exonuclease activity from the MRX complex. Cleavage in the ssDNA region was dependent on the nuclease activities of both the MRX and Sae2 complexes (Figure 3.10A). This was further confirmed when cleavage at the ssDNA gap was not observed in the presence of nuclease deficient MR complexes, M(D16A)R and Mre11-3/R (Figure 3.10B). This suggests the Mre11 complex exonuclease activity may be required to increase the length of the ssDNA region adjacent to a hairpin-capped end, which would facilitate Sae2 endonuclease activity on the ssDNA in the gap (Figure 5.1). This would result in removal of the hairpin from the DNA ends. Lobachev et al. demonstrated that inverted *Alu* repeats embedded in the *LYS2* gene are entirely

lost when recombination occurs correctly (Lobachev et al., 2002). To investigate where the hairpin structure is cleaved in vivo we plan to construct a gene with a portion duplicated and embedded into the gene in the inverted position. Homologous DNA including one repeat sequence alone and with flanking regions would be constructed to look for homologous recombination between the homologous sequences. If the hairpin structure is entirely removed the inverted repeat will no be able to recombine with donor sequences which only include the repeat sequence. Also, linear plasmid DNA containing a marker and hairpin-capped ends will be transformed into *Δrad52* strains. Hairpin-capped DNA ends will block homologous recombination and NHEJ can join DNA ends which have been processed by MRX and Sae2 complexes. Where MRX and Sae2 complexes cleave the hairpin-structure can partially be determined by analysis of the sequences at the joints of the recovered product.

SAE2 EXHIBITES SSDNA ENDONUCLEASE ACTIVITY

To characterize Sae2 ssDNA endonuclease activity in greater detail we tested several branched substrates. The Sae2 protein cut at several sites in the ssDNA region of the 5' flap, predominantly at the single-strand/double-strand junction, along with minor cleavage sites in the duplex region of the branched substrate and removal of the most 5' nucleotide (Figure 3.11A). Nuclease activity

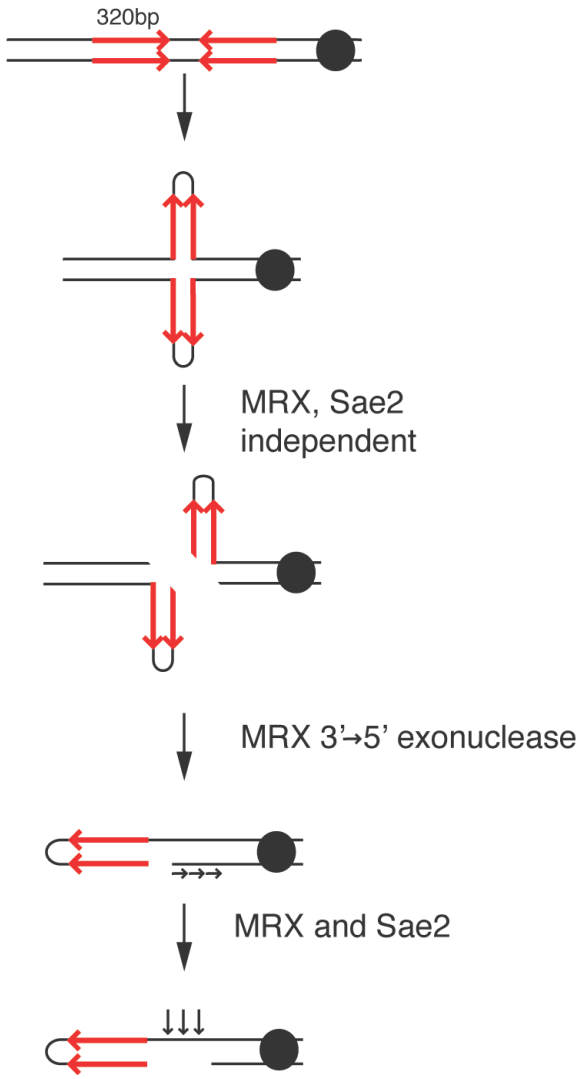

Figure 5.1 Model for hairpin removal. As shown in Figure 1.4 large inverted repeats can form cruciforms. In the absence of MRX or Sae2, hairpin-capped ends lead to duplication of the inverted repeats. In the presence of MRX and Sae2 the hairpin is removed. After the cruciform base is cleaved it is likely that there is a nick or gap adjacent to the hairpin. Based on our in vitro data, we propose that MRX 3'→5' exonuclease activity increases the gap and that MRX stimulates Sae2 cleavage in the ssDNA region thus removing the entire hairpin.

was altered when complementary strands were introduced to either flap. Cleavage of 5' flap was blocked when a fully paired complementary strand to the 5' flap was present, although in this case, cleavage in the duplex region increased (Figure 3.11B). Cleavage was never entirely blocked by any of the complementary strands annealed to the flaps on the branched substrate (Figure 3.11B). Although the Sae2 protein predominantly exhibits ssDNA endonuclease activity the protein is able to duplex DNA. Also, the Sae2 protein was able to cut the ssDNA in the 3' flap, in the middle of the flap rather than at the base (Figure 3.11C). We were unable to determine if there is any sequence specificity involved, but this is unlikely considering the diverse oligonucleotide sequences used.

NUCLEASE ACTIVITY OF THE SAE2 MUTANTS

We designed several mutant Sae2 proteins to investigate domains essential for protein-protein interaction, DNA-protein interactions, and protein function. The Sae2(ΔC) protein was truncated at the C-terminal end, deleting amino acids 251-345. Similarly, the Sae2(ΔN) protein was constructed by removing amino acids 21-172 using in-frame *SacI* restriction sites. The Sae2(G270D) protein was discovered in a random mutagenesis screen for mutants defective in resolving hairpin-capped DSBs (A. Rattray, personal communication) (Rattray et al., 2001). The Sae2(5A) and Sae2(5D) mutant

proteins were based on putative Tel1/Mec1 phosphorylation sites S73, T90, S249, T279, and S289 identified by Longhese and colleagues (Baroni et al., 2004). We constructed mutants in which the serines or threonines were mutated to alanines or aspartates for the Sae2(5A) and Sae2(5D) proteins, respectively. The aspartate changes in the Sae2(5D) mutant were designed to mimic phosphorylation. Although mammalian homologs for the Sae2 protein have yet to be identified there are several putative Sae2 proteins in other fungi. S279 and S289 phosphorylation sites are in a conserved region in the C-terminus (Figure 3.2). Interestingly, all the mutant Sae2 proteins showed identical profiles when passed through the gel filtration column except for the Sae2(ΔN) protein (Figure 3.1D). Gel filtration profiles suggest the Sae2 protein forms different multimeric complexes in vitro. We plan to investigate the in vivo multimeric state of the Sae2 protein by expressing MBP-Sae2 under control of an endogenous promoter. The MBP-Sae2 protein will be partially purified by ion-exchange chromatography and multimeric forms will be determined by passing the protein through a gel-filtration column.

The Sae2(5D), Sae2(5A) and Sae2(ΔC) mutant proteins exhibit DNA binding activity similar to wildtype (Figure 3.4A and Table 5.1). The

Table 5.1

	DNA binding	MRX-DNA binding	ssDNA endo-nuclease activity	hairpin endo-nuclease activity	MMS survival	*LYS2* conversion
Sae2 wt	+++	+++	+++	+++	+++	+++
Sae2(G270D)	-/+	N/A	-/+	-	+	-
Sae2(5D)	+++	+++	+++	+++	+	-
Sae2(5A)	++	-	++	-	+	-
Sae2(ΔN)	-	-	-	-	+	-
Sae2(ΔC)	++	+++	++	-	+	-

Sae2(G270D) protein exhibited weak DNA binding while the Sae2(ΔN) exhibited complete loss of DNA binding activity. This suggests the N-terminal domain is

required for efficient DNA binding. Although, diminished DNA binding of the Sae2(G270D) mutant suggests regions other than the N-terminus are also important for DNA binding.

Since wildtype Sae2 was able to form higher order DNA-protein complexes with the MRX complexes, we investigated whether these complexes were due to Sae2-MRX interaction or both complexes separately binding the dsDNA substrate. Both the Sae2(5D) and Sae2(ΔC) mutants were able to supershift the MRX-DNA complex; although, neither the Sae2(5A) nor Sae2(ΔN) were able to form higher complexes (Figure 3.4B). Interestingly, both the Sae2(5D) and Sae2(5A) proteins were able to binding DNA independently, but the Sae2(5D) mutant was able to form higher complexes with MRX and DNA while the Sae2(5A) mutant was not (Figure 3.4B). This suggests that the Sae2 and MRX complexes are interacting together on the DNA and not just binding separately to the same DNA fragments. Interestingly, Mre11 and Sae2 colocalize to DSB foci in vivo regardless of Mec1/Tel1 phosphorylation of the Sae2 protein (Lisby et al., 2004). Presumably our recombinant Sae2 protein is not phosphorylated when expressed in *E. coli* and we show here that it is still able to interact with the MRX-DNA complex. In contrast, the Sae2(5A) protein does not form higher-order complexes with MRX (Figure 3.4B). The point mutations in the Sae2(5A) protein are probably disrupting not only Sae2 phosphorylation but also MRX-Sae2 interaction. Lastly, the Sae2(ΔC) protein is able to form higher order

complexes with MRX and DNA, showing that the C-terminus of Sae2 is dispensible for MRX and Sae2 interaction (Figure 3.4B).

As shown in Chapter 3, the Sae2 protein exhibits endonuclease activity on ssDNA and is stimulated by the MRX complex to cleave ssDNA adjacent to DNA hairpin substrates (Figure 3.9A). The Sae2 mutant proteins were utilized in these nuclease assays to determine which domains in the protein are essential for these activities. The Sae2(ΔN) protein exhibits no DNA binding or nuclease activity, although the lack of nuclease activity may be a result of no DNA binding activity (Figure 3.9B). The Sae2(ΔC) protein exhibits DNA binding and endonuclease activity independent of the MRX complex. Although the Sae2(ΔC) protein is able to form higher order complexes with the MRX complex and DNA, the MRX complex is not able to stimulate the Sae2(ΔC) protein to cleave the ssDNA adjacent to a DNA hairpin (Figure 3.9B). This data suggests the C-terminus is required for cooperative activities between MRX and Sae2 on hairpin substrates. Possibly the C-terminus is required for MRX stimulation of the Sae2 protein, since the Sae2(ΔC) protein exhibits endonuclease activity independently of the MRX complex but is not stimulated by the MRX complex to cleave ssDNA adjacent to a hairpin. It is also possible that the C-terminus may be specifically involved in hairpin recognition; this is currently being tested in gel mobility shift assays in vitro.

The Sae2(5A) protein exhibits DNA binding and ssDNA nuclease activity but is not stimulated by the MRX complex to cleave ssDNA adjacent to hairpin structures (Table 5.1). Also, the Sae2(5A) protein does not form higher order complexes with the MRX complex and DNA, while the Sae2(5D) protein can form these complexes. This suggests the putative Mec1/Tel1 phosphorylation sites mutated to alanines disrupt MRX-Sae2 interaction, although mutation of these sites to mimic phosphorylation allow for MRX-Sae2 interaction. The last putative phosphorylation site, S289, is in a block of amino acids that is conserved among the putative Sae2 proteins (Figure 3.2). Therefore, the Sae2(5A) protein possibly lacks nuclease activity stimulated by the MRX complex because protein-protein interaction is lost. The Sae2(5D) protein is the only mutant that exhibits activity is all of our in vitro assays. We incubated wildtype Sae2 protein with phosphatase to remove any phosphorylation from the *E. coli* expression system and ATM to phosphorylate the putative SQ/TQ sites; however, neither enzyme altered the activity of the Sae2 protein (data not shown). This suggests that phosphorylation of Sae2 in vivo may affect Sae2 signaling and not nuclease activity or interaction with the MRX complex.

Although the Sae2 mutant proteins exhibited different activities in the in vitro assays, all mutant proteins exhibited intermediate sensitivity to MMS when compared to the wildtype and *Δsae2* strains (Figure 3.12). In vivo phenotypes correlate with in vitro except for the Sae2(5D) mutant. One possibility is that the

Sae2(5D) mutant protein is not stable and is unable to function in vivo. To verify equal protein expression we plan to determine the levels of each mutant protein in vivo.

SAE2 MUTANTS ARE DEFECTIVE IN MMS SURVIVAL AND HAIRPIN PROCESSING IN VIVO

The Sae2 protein is phosphorylated during S/G_2 phase and has been shown to negatively regulate checkpoint activation in response to DNA damage (Clerici et al., 2005a). Overexpression of the Sae2 protein prevents checkpoint activation, while in Sae2(5A) and *Δsae2* strains, cells fail to recover from cell cycle checkpoint arrest (Clerici et al., 2005a). The Sae2(5A) protein, as previously shown, exhibited intermediate sensitivity to MMS. Considering our in vitro data, the deficiencies in DNA repair observed with the Sae2(5A) mutant may be due to lack of Sae2 and MRX interaction rather than to a lack of phosphorylation. Interestingly, the Sae2(5D) protein also exhibited intermediate sensitivity to MMS even though it showed activity in all in vitro assays (Figure 3.14). If the levels of the Sae2(5D) protein are equivalent to wildtype in vivo, it could be that the Sae2(5D) protein prevents cell cycle checkpoint activation after DNA damage, which leads to inefficient DNA repair and sensitivity to DNA damaging agents. Constant Sae2 phosphorylation may lead to the persistence of the MRX complex at the DSB foci and stalled DNA repair intermediates. To test

this hypothesis, we would need to track Mre11 foci formation in wildtype and Sae2(5D) strains.

Using the cruciform recombination assay in vivo, we also observed that all the Sae2 mutants exhibited deficiencies in resolving hairpin-capped ends similar to *Δsae2* strains. The Sae2(5A), Sae2(G270D), Sae2(ΔN) and Sae2(ΔC) proteins were all deficient in MRX dependent hairpin cleavage in vitro and, not surprisingly, these Sae2 mutants were unable to resolve hairpin-capped DNA ends in vivo (Table 3.1). Unexpectedly, the Sae2(5D) mutant, which was active in all of our in vivo assays, was also deficient in in vivo hairpin resolution (Table 5.1). As discussed above, the Sae2(5D) mutant may alter cell cycle arrest and therefore inhibit efficient repair.

Resolving hairpin-capped DSBs has been demonstrated to be essential for the maintainence of genomic stability in organisms ranging from *E. coli* to humans. Failure to resolve such DSBs, caused by inverted repeats, can lead to gene amplification. In *E. coli*, inverted repeats are efficiently resolved in the presence of the SbcC/SbcD complex. Similarly in yeast, deletion of *MRE11*, *RAD50, XRS2* or *SAE2* stabilizes inverted repeats and increases genomic instability. Small inverted repeats (4-9 nucleotides) can be found in almost any gene and are potentially unstable when adjacent to a DSB (Rattray et al., 2005). Foldback of inverted repeats at a DSB can form a hairpin structure and prime DNA synthesis from the DNA end, leading to gene amplification (Rattray et al.,

2005). These small inverted repeats can initial several rounds of break-induced gene amplification, thus increasing genome instability. The MRN complex likely plays an important role in reducing the frequency of these events in human cells.

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